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PREGNANCY AS A PRECIPITATING FACTOR IN DIETETIC LIVER NECROSIS IN RATS.

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MASSIVE hepatic necrosis has long been regarded as a condition particularly liable to occur in pregnancy and during lactation (Frerichs, 1860; Hunter and Spriggs, 1908; Rolleston and McNee, 1929; Peller, 1936; Eppinger, 1937). Many such cases occur in association with infective hepatitis (Mossé, 1879; Hardie, 1889–90; Hayward, 1889–90; Cockayne, 1912), but there are a number in which no such association can be demonstrated, and it remains an open question whether pregnancy alone can cause such necrosis. As long ago as 1879 Mossé distinguished a condition of *ictère grave aggravée*, which he defined as a simple "catarrhal jaundice" which was aggravated by malnutrition or pregnancy into becoming a massive hepatic necrosis. When one considers that foetal tissues must be formed from the nutriments ingested by the mother it is evident that the influence of pregnancy and dietary deficiency upon maternal nutrition may be similar. That a combination of these two factors might lead to massive necrosis in patients with infective hepatitis was suggested by Himsworth and Glynn (1944*a*) and by Nixon, Egeli, Laqueur and Yahya (1947).

By means of a low protein diet, Himsworth and Glynn produced necrosis of the liver in rats, histologically closely resembling the acute atrophy which occurs in man and in pregnant woman; they correlated this experimental liver injury in the rat with the deficiency of sulphur-containing amino-acids, especially methionine (Himsworth and Glynn, 1944*b*, *c*; Glynn and Himsworth, 1944). Later it was found that a double dietary deficiency was required to produce this lesion, and that the rat must be depleted not only of sulphur-containing amino-acids but also of tocopherol (Schwartz, 1944; György, 1947; Himsworth and Lindan, 1949).

The liability to develop liver necrosis on a regime low both in tocopherol and in sulphur-containing amino-acids ("necrogenic diet") had been shown to

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decrease with age (Lindan and Himsworth, 1950). On weaning rats were very susceptible to such double deficiency; in older animals the liver lesion developed only after a delay of many weeks and the percentage of survival was high. The requirements of essential nutriments during pregnancy resemble those of young fast-growing animals. A diet just sufficient for the maintenance of an adult non-growing organism may become highly deficient during the later period of gestation when the basal metabolism and rate of anabolic processes with new tissue formation are increased. It is important to realise that both nutrients, deficiency of which leads to experimental liver necrosis, play an essential role in gestation. Tocopherol is indispensable for normal gestation in the rat, its absence leading to death and resorption of foetuses (Evans and Bishop, 1922). When the animal has only a little of this vitamin available the question arises, to which protective function of tocopherol will priority be given, safety of foetuses or protection of the liver? A similar question can be asked concerning the minimal protein requirements for gestation (Goettsch, 1949), and for protection against liver lesions. The present experiments were therefore undertaken to investigate the effect of nutritional deficiency of protein and tocopherol upon liver necrosis in relation to pregnancy.

EXPERIMENTAL.

Animals.

Albino (Wistar strain) female rats were used. When mated they were left with the bucks for 7 days. The rats which delivered normally (except those in Experiments 5 and 6) were sacrificed shortly after delivery; those that became ill during pregnancy were killed when moribund. The uterus was examined for number of normal and resorbing foetuses. The normal foetuses were removed from the uterus and weighed. The number of young born and the weight of the whole litter were recorded. Non-pregnant controls were killed after the experimental period of 30 or 40 days on the yeast diet.

Diets and supplements.

Two experimental diets were used, both deficient in tocopherol, but differing in their protein composition, one containing 25 per cent casein, the other 7 per cent yeast protein supplied from dried baker's yeast (obtained from the Distillers' Co., Ltd., Edinburgh). The casein diet was considered to be deficient in tocopherol only, the other diet which was also low in protein (referred to as "necrogenic yeast diet") was doubly deficient—in tocopherol and in sulphur-containing amino-acids (Lindan and Himsworth, 1950). The amino-acid composition and protein inadequacy of the necrogenic yeast diet has been described by Lindan and Work (1951*a*, *b*). The composition of both deficient diets is shown in Table I. The olive oil and cod liver oil were mixed daily into the diets, as were the water-soluble vitamins. All diets were fed *ad lib.*

Tocopherol content of deficient diets.

Tocopherol, estimated chemically (method of Tosic and Moore, 1945) in casein, corn starch and olive oil, amounted to 1·1 mg. per 100 g. of both diets, the olive oil providing 1 mg. The presence of this small amount of tocopherol was also confirmed biologically in the casein diet. Although the female rats

reared on the casein diet grew well and littered normally, their young at the end of the lactation period showed symptoms of tocopherol deficiency (convulsions and paralysis of hind legs). The amount of tocopherol in the casein diet was therefore sufficient for normal growth and gestation of the mother, but was inadequate to ensure normal supplies to the litter (Lindan and Himsworth, 1950).

TABLE I.—*Composition of Deficient Diets.*

	Casein diet, deficient in tocopherol (per cent).	Necrogenic yeast diet, deficient in tocopherol and in sulphur-containing amino-acids (per cent).
Casein (Glaxo " C ")	25	—
Yeast, dried baker's	—	18 (= 7 per cent protein)
Corn starch	66	73
Salt mixture (Glaxo)	3	3
Olive oil (B.P.)	5	5
Cod-liver oil	1	1

To these diets the following supplements of water-soluble vitamins were added per rat/day: Thiamin hydrochloride 20 μg., pyridoxin 20 μg., riboflavin 25 μg., calcium pantothenate 100 μg.

In order to confirm the protective action of tocopherol against liver necrosis, DL-α-tocopheryl acetate (33 per cent solution in arachis oil (Roche Products, Ltd.)) was given by mouth to some rats.

RESULTS.

Normal Gestation and Delivery in Rats Fed the Necrogenic Yeast Diet.

Experiment 1.—Adult normal rats were given the necrogenic yeast diet and immediately mated. The rats which became pregnant delivered between the 24th and 30th day on the yeast diet. The uteri showed the usual picture after delivery with no signs of resorption. The livers of these pregnant rats and of the non-pregnant rats, killed after 40 days on the yeast diet, were not necrotic (Table II, Exp. 1).

This experiment showed that the necrogenic yeast diet lowered the fertility rate; only 9 out of 23 rats left with the bucks for 7 days became pregnant, but these rats were able to produce normal litters and did not develop liver necrosis. Because the animals were well fed during the pre-experimental period, their bodies could have stored enough tocopherol and protein for normal gestation and protection against liver necrosis during 30 days on the deficient diet. The next experiments were therefore devised to deplete the rats of their tocopherol and protein stores, and to determine the effect of pregnancy on these depleted animals fed the necrogenic yeast diet.

The Effect of Preparatory Tocopherol Depletion on Subsequent Development of Liver Necrosis in Pregnant Rats.

Experiment 2.—In order to lower the normal body stores of tocopherol, normal rats reared on stock diet were fed the casein diet for 60 days. They were then given the necrogenic yeast diet and mated. The rats which became pregnant

littered normally. The uteri and the liver of all rats, pregnant and non-pregnant, were normal, showing that the 60-day preparatory period on casein diet did not seriously deplete the rats of their tocopherol stores (Table II, Exp. 2).

Experiment 3.—Experiments on weanling rats (Lindan and Himsworth, 1950) have proved that the tocopherol content of the diet during lactation is of great importance for the subsequent development of liver necrosis in young rats. In the present experiment an attempt was therefore made to produce necrosis in pregnant rats reared on the low-tocopherol diet (Mason and Bryan, 1938).

The young rats were born of stock breeding rats maintained on the stock diet throughout pregnancy and until the 10th day after delivery, when the young opened their eyes. Thereafter the casein diet was given. After weaning at 21 days the young were maintained on the same casein diet until they were 60–120 days old. Out of 64 females prepared in this way, then given the necrogenic yeast diet and mated, only 20 became pregnant. Four of them littered (1–6 young per litter), the uteri being normal in two rats and showing resorbing foetuses (4 and 7) in the other two. The remaining 16 pregnant rats developed liver necrosis at the end of pregnancy and died or were killed when ill. Examination of uteri showed that in the uterus of one rat there were 12 old resorbing foetuses and no normal foetuses. The uteri of the other rats contained 3–11 foetuses, and in five cases there were also some resorbing foetuses (1–3 in a litter). When the uterus was removed immediately after killing the animal, the normal foetuses were found to be alive. The livers of these normal foetuses were not necrotic, liver necrosis occurring only in the mother rat. The non-pregnant rats, killed after 40 days on the yeast diet, showed normal livers (Table II, Exp. 3).

A control group of 8 rats, reared on the casein diet as before and subsequently transferred to the necrogenic yeast diet and mated, was given tocopherol (20 mg. twice a week) when on the yeast diet. Rats which became pregnant littered normally, and the livers of all rats, pregnant and non-pregnant, were normal (Table II, Exp. 3).

Experiments 1–3 demonstrate the importance of body tocopherol stores in the prevention of liver damage. Dietetic liver necrosis was precipitated by pregnancy provided that the animal had been subjected to a preparatory tocopherol depletion.

The Effect of Preparatory Protein Depletion upon Subsequent Development of Liver Necrosis in Pregnant Rats.

Experiment 4.—It has been shown that in addition to tocopherol depletion, the quantity and quality of protein fed in the preparatory period also has an influence on the survival of rats subsequently given necrogenic yeast diet (Lindan and Himsworth, 1950). The following experiment was planned to produce liver necrosis in pregnant protein-depleted animals.

Adult rats were fed the casein diet for 30 days and then were given the necrogenic yeast diet for another 30 days. Thus the animals were on tocopherol-deficient diets for 60 days altogether, and during the second half of this time they were also on diet low in sulphur-containing amino-acids. They grew normally when on the casein diet, but during the 30 days on the yeast diet either did not grow or lost up to 15 g. in weight. After this preparatory period the animals

were mated and continued on the necrogenic yeast diet. Only 3 out of 10 rats became pregnant; two died of liver necrosis, and one littered normally (4 young) and had a normal liver. The uterus of one dead rat contained 9 normal foetuses, and in the other uterus there were 5 normal foetuses and one resorbing. The non-pregnant rats killed after a total of 60 days on the yeast diet had normal livers (Table II, Exp. 4).

The low fertility rate of protein-depleted animals resulted in only a small number of pregnancies during the experimental period on the yeast diet. Nevertheless, the results suggest that the protein composition of the diet during the preparatory period has an effect upon the subsequent development of liver necrosis in pregnant rats.

The Acceleration of Preparatory Nutritional Depletion by Pregnancy.

Experiment 5.—A group of 18 rats, reared on stock diet (analogous to the group of Exp. 2), was mated when transferred to the casein diet. In contrast to the rats fed the necrogenic yeast diet, all of them littered, producing 108 normal young, which were left with their mothers. The outcome of lactation was as follows: 3 litters (comprising 14 young) were not suckled at all and died, 2 litters (10 young) were weaned normally after 21 days without any loss, and the other 13 litters (84 young) showed symptoms of tocopherol deficiency (convulsions, paralysis of hind legs and death) between the 18th and 20th day of the lactation period.

Four of these tocopherol-deficient litters (25 young) perished, and only 39 out of 59 were weaned in the remaining 9 litters. This heavy loss of young before weaning shows that the casein diet with its low tocopherol content was not adequate for normal development. The young usually open their eyes between the 10th and 15th day of life, and they start taking food found in the cage in addition to maternal milk. In this experiment the tocopherol contents of maternal milk and the casein diet were not sufficient to meet the demands of the young.

These mother rats, after this preliminary period of 60 days on casein diet, during which pregnancy and lactation occurred, were then given the necrogenic yeast diet and mated again. The rats which did not become pregnant were killed after 40 days on the yeast diet and had normal livers. This non-pregnant group included one rat which in the preliminary pregnancy did not suckle her young, and one which lost the whole litter at the end of lactation through tocopherol deficiency. Out of the 11 pregnant rats one delivered and ate the young; its uterus showed signs of seven delivered foetuses and its liver was normal. At the end of pregnancy (after 20–27 days on the yeast diet) the other 10 rats became suddenly ill and died or were killed when moribund. Two of them were delivering but obviously could not accomplish the parturition. The livers of these 10 rats showed massive necrosis. Examination of the uteri showed that the total number of normal and resorbing foetuses was 3–8 per litter, partial resorption occurring in two cases.

A control group of 8 rats, was given 3 doses, total 150 mg., of tocopherol by mouth during their first pregnancy on casein diet. The young born to these mothers were all properly suckled and weaned. The mother rats were then transferred to the yeast diet and mated again. The rats which became pregnant a

second time littered normally. The livers of all rats, pregnant and non-pregnant (killed after 40 days on the yeast diet), were normal, and the uteri of the pregnant rats showed no resorption signs (Table II, Exp. 5).

Comparison of the results of Exp. 5 with those of Exp. 2 leads to the following conclusions: First, that it was not possible to deprive the normal adult rat of its body tocopherol stores simply by giving the casein diet for as long as 60 days. Secondly, that tocopherol deficiency developed on the same casein diet during the same period if pregnancy occurred. Thus, in pregnancy more tocopherol was used than was provided by the casein diet, and the mother rat's store became depleted; symptoms of tocopherol deficiency occurred in the suckled young. If these depleted mother rats again became pregnant when on the necrogenic yeast diet, liver necrosis developed. Thirdly, supplementing the casein diet with 150 mg. of tocopherol during the gestation period prevented loss of young and protected the mother rat against liver necrosis during her second pregnancy when fed the necrogenic yeast diet.

TABLE II.—*Effect of Diet and Pregnancy on Liver Necrosis.*

No. of exp.	Number of rats.	Preparatory period. Days on diet. Stock diet.	Casein diet.	Tocopherol suppl.	Yeast diet.	Experimental period 20–40 days on yeast diet. Tocopherol suppl.	Number of animals. Non-pregnant. Total.	Non-pregnant. With liver necrosis.	Pregnant. Total.	Pregnant. With liver necrosis.
1	23	60–120	0	–	0	–	14	0	9	0
2	42	60	60	–	0	–	24	0	18	0
3	64	0	60–120	–	0	–	44	0	20	16 (80%)
	8			–		+	3	0	5	0
4	10	60	30	–	30	–	7	0	3	2
5	18	60	60	–	0	–	7	0	11	10 (91%)
	8		First pregn.	+		–	4	0	4	0
6	12	60	30	–	30 First pregn.	–	8	0	4	4

Experiment 6.—Rats, reared on stock diet, were fed the casein diet for 30 days and then were given the necrogenic yeast diet and mated. They were not allowed to suckle their young, which were destroyed immediately after birth. The rats were mated again after the 30 days on the yeast diet and were continued on the same diet. Thus, they were prepared in a similar way to the rats of Exp. 4, except that they went through a normal pregnancy during the first 30 days on the yeast diet. Four became pregnant for a second time and developed liver necrosis. Three uteri had normal foetuses and one uterus had 3 normal and 3 resorbing foetuses. The rats which did not become pregnant a second time, killed after 60 days in all on the yeast diet, had normal livers (Table II, Exp. 6).

If pregnancy occurred during the preparatory period either on casein (Exp. 5) or on yeast diet (Exp. 6) a very high incidence of liver necrosis resulted during

the subsequent pregnancy on the yeast diet. It may therefore be concluded that pregnancy on a deficient diet, although it may not immediately produce liver necrosis, strongly predisposes to the development of a fatal liver lesion during subsequent pregnancies on a similar or worse diet.

Effect of Dietary Depletion upon Foetal Growth and Changes in Body Weight of Pregnant Rats in Relation to Incidence of Liver Necrosis.

Foetal growth.—The total number of foetuses (normal and resorbing) was very variable (ranging from 1 to 12) for individual pregnancies, but on an average it was similar in all experiments, pointing to the fact that the implantation rate was not influenced by diet and that the incidence of liver necrosis was not limited to rats with either small or large litters. Eleven cases of resorption (10 partial and 1 complete) were found in a total of 85 pregnant rats on the yeast diet. The percentage of pregnancies with resorption increased with dietary depletion. As resorption occurred in small and large litters it was not related to the litter size. Because of the inconstant number of foetuses and variable weight of individual foetuses in a given pregnancy, the total weights of the litters were also variable (range 5–65 g.). The development of liver necrosis was not related to the weight of the whole litter or to the mean weight of individual young.

Changes in body weight of pregnant rat.—The change in weight of the mother rat was the overall result of the development of foetal tissue (total weight of litter) and development or regression of maternal tissues. The term " maternal tissues " represents here the difference between the total weight of pregnant rat and the total weight of litter and includes the placental tissues and amniotic fluid. The total gain in weight by the normal pregnant rat always exceeds the total weight of the litter, showing that the rat is able to build foetal tissue and at the same time to increase its own tissues. Subtraction of the weight of normal foetuses from the weight of the mother rat shows that the majority of pregnant rats fed the yeast diet produced their young at the expense of other body tissues. This loss in weight of maternal tissues was again very variable, and could not be related to the number of young in a litter or to the incidence of liver necrosis.

DISCUSSION.

Two main points which emerge from the results are: first, that pregnancy is a precipitating factor in dietetic liver injury, and that the nutritional status of an animal before gestation is of importance; second, that tocopherol requirements are variable throughout the life of an animal and depend on the dietary protein level.

Within the short experimental period of 30 days, dietetic liver necrosis did not occur in adult rats unless they were pregnant, and then only in those pregnant rats which had low body stores of tocopherol. To obtain such low body stores the rats either had to be reared on a low-tocopherol high-protein diet from the 10th day of lactation, or have their normal tocopherol stores lowered by preparatory depletion after rearing on a normal diet.

Three methods of depletion were investigated. The first, feeding casein diet for 60 days, was unsuccessful; apparently the tocopherol stores were not sufficiently lowered because the requirements were not high and were met by the

tocopherol in the diet. The second method involved a pregnancy, with or without lactation, during the preparatory period of 60 days on the casein diet. This was successful, resulting in the reduction of the rat's tocopherol stores, and thus demonstrating that the vitamin E requirements during pregnancy were increased and could not be met by the tocopherol in the casein diet. Successful depletion of tocopherol also resulted from a preparatory period of 30 days on the casein diet and another 30 days on the yeast diet. The tocopherol stores were diminished in this way, but there was also some protein depletion as indicated by loss in body weight. A combination of two methods of tocopherol depletion, consisting of a " preparatory pregnancy " during the 30 days on the yeast diet, had the most marked effect of all on body tocopherol depletion.

The inter-relation between tocopherol and protein metabolism in rats is already well established (Dam, 1944; Hove, 1946; Moore, 1949; Granados, Aaes-Jørgensen and Dam, 1950). Experimental diets used in this laboratory, the 25 per cent casein and 7 per cent yeast protein diets, each contained 1·1 mg of D-α-tocopherol per 100 g. This level of tocopherol with the high protein (25 per cent casein) diet was biologically nearly adequate, as judged by normal growth of rats and normal gestation; only lactation was unsatisfactory. On the low protein (7 per cent yeast protein) diet a level of 1·1 mg. per cent of D-α-tocopherol was not sufficient to prevent liver necrosis. Schwartz (1948), using diets with yeast supplying the majority of proteins, found that supplements of 5 mg. D-L-α-tocopherol per 100 g. diet prevented the liver lesions. In the present experiments when tocopherol was fed to rats in order to protect them against liver necrosis, the protective doses given by Schwartz were exceeded.

It seems that tocopherol, like many other nutrients, when available only in small amount, is used rather for protection of the embryos than of the mother. Out of a total of 38 rats which had low body stores of tocopherol and were pregnant when on the yeast diet, 32 developed liver necrosis (84 per cent) and only 11 showed foetal resorption (29 per cent). However it should be pointed out that the death of resorbing foetuses occurred before development of liver necrosis. The development of liver necrosis was not related to the total weight of newly formed embryonic tissue, since it occurred in rats with 3 to 11 embryos; neither was it related to the changes in weight of maternal tissues. Necrosis, if it developed, only occurred during the latter part of gestation when the metabolic rate of the pregnant organism increases (Rowe, Gallivan and Mathews, 1930) and when total protein synthesis is highest (Huggett, 1941).

The liver is the organ showing the greatest rate and degree of protein changes during protein starvation (Addis, Poo and Lew, 1936) and during the physiological stress of pregnancy (Poo, Lew, Lee and Addis, 1940). Normally it contains protein stores which are easily depleted during protein deficiency, thus protecting other organs. The liver also stores considerable amounts of tocopherol when the supply of this vitamin is abundant. After feeding a tocopherol-deficient diet the liver similarly contains very little vitamin E in comparison with other organs, suggesting a protective function of the liver against tocopherol deficiency in other organs (Mason, 1944). The ease with which the liver is depleted of its protein and tocopherol stores must increase during pregnancy when the foetuses enjoy nutritional priority over the mother. This explains the role of pregnancy in precipitating dietetic liver necrosis in the mother rat and the absence of similar lesions in the foetus.

SUMMARY.

Pregnancy precipitated the onset of massive liver necrosis in rats fed a diet low in both tocopherol and sulphur-containing amino-acids (" necrogenic diet "). Liver necrosis developed during the later part of gestation in animals which had been fed the necrogenic diet for only 20–30 days, while non-pregnant controls did not develop liver lesions in 30–40 days.

An adequate intake of tocopherol during the pre-experimental period (resulting in large body stores of vitamin E) protected the rats against liver lesions when they subsequently became pregnant while receiving the necrogenic diet.

Pregnancy increased the demand for tocopherol regardless of the protein content of the diet. This demand was higher when the diet was deficient in sulphur-containing amino-acids.

When the uterus was removed immediately from a moribund rat with liver necrosis, the foetuses were found alive. Although the dietary depletion of pregnant rats resulted in maternal liver necrosis and sometimes partial resorption of her litter, no necrosis was observed in the foetal livers.

The incidence of liver necrosis in pregnant rats was not related to the litter size, total weight of litter or partial foetal resorption.

Great variability in changes in body weight were observed in pregnant rats fed the necrogenic diet. This is discussed in relation to the incidence of liver necrosis, litter size and weight.

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METHIONINE AND CHOLINE DEFICIENCY IN THE RAT WITH SPECIAL REFERENCE TO THE PREGNANT STATE.

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THE relationship of choline and methionine in nutrition and the concept of the labile methyl group as an essential dietary constituent has been established by the work of numerous investigators, notably by du Vigneaud and his collaborators (du Vigneaud, 1948).

These biochemical investigations establishing the key position of methionine in the nutrition of the rat and of man, and those of Whipple (1942), which demonstrate clearly the protective value possessed by methionine for the livers of protein-depleted dogs, suggest that further investigation of methionine deficiency, especially with a co-existent deficiency of choline, might be profitable in circumstances such as pregnancy, in which metabolic requirements are likely to be high.

Much of the earlier work on the liver lesions associated with a low protein diet suffered from the disadvantage that the diets used were not composed of purified ingredients, and the precise nature of the deficiency was frequently in doubt. More recently, the pathological features of a pure methionine deficiency have been described consisting of loss of weight, anaemia and hypoproteinaemia (Glynn, Himsworth and Neuberger, 1945; Albanese, 1946). The principal changes associated with a deficiency of choline are well known (a review by Griffith (1941) presents the main features), but in most of this work appreciable amounts of methionine were present in the diet and there was no true deficiency of labile methyl groups.

The experiments here described were carried out as a preliminary study in the investigation of the metabolism of methionine and choline in normal and toxaemic human pregnancy (Sims, 1952). Their object was to determine whether a state of depletion of labile methyl groups is reflected in the blood, urine, and tissue concentrations of methionine and choline; to discover whether in pregnant animals there is evidence of a heightened demand for these substances, and to study the lesions occurring in this deficiency. It seemed desirable also to determine whether in severe methionine-choline deficiency there is a low methionine content of the plasma and tissue proteins.

EXPERIMENTAL DETAILS.

Assay of choline and methionine.

Choline and methionine were measured by the standard methods of microbiological assay. For choline the technique of Horowitz and Beadle (1943) was used with minor modifications, the organism being the choline-less mutant of

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Neurospora crassa, while methionine was assayed using *Leuconostoc mesenteroides* P60, after the method of Lyman, Moseley, Butler, Wood and Hale (1946) and Barton-Wright and Curtis (1948). The free methionine in urine was measured on the diluted but otherwise untreated specimen (urine specimens were collected under toluene), and the free methionine of blood plasma on the diluted specimen from which the protein had been removed by heat coagulation at pH 4·7 (Harper, Kinsell and Barton, 1947). The choline content of urine was measured on specimens prepared by hydrolysis with 2 N sulphuric acid for 5 hours at 15 lb. pressure and subsequent removal of the excess acid by precipitation with barium hydroxide. This procedure destroyed the interfering substances and permitted full recovery of choline added to the final assay preparation. There was a loss of choline of 10–20 per cent due to adsorption on the barium sulphate precipitate, but this method nevertheless proved more satisfactory than purification by adsorption on permutit.

The assay of the total methionine and choline of plasma and tissues was carried out on a preparation obtained by the hydrolysis of a known amount (1 ml. plasma or approximately 0·5 g. fresh tissue) with 12 ml. 2 N HCl for 6 hr. at 15 lb. pressure, filtration, neutralisation and subsequent dilution.

Experimental animals.

The rats used were white " Wistar " strain in-bred animals, and the experimental and control groups were made up from litter mates arranged in pairs which as far as possible corresponded in size, one of each pair being placed in the experimental group and one in the control group.

Diet.

The experimental diets were composed of purified ingredients, whole casein or casein hydrolysate being used as a source of protein, lard as the source of fat, and sucrose for the supply of carbohydrate. Inorganic salts and pure vitamins were added.

The basal diet was made up as follows: Casein 10–20 g. in different experiments, sucrose 58–48 g., lard 25 g., salt mixture 6 g., calcium carbonate 1 g., water soluble vitamins plus or minus choline 10 ml., fat soluble vitamins 1 g. The casein used was ordinary commercial light white soluble material for base-

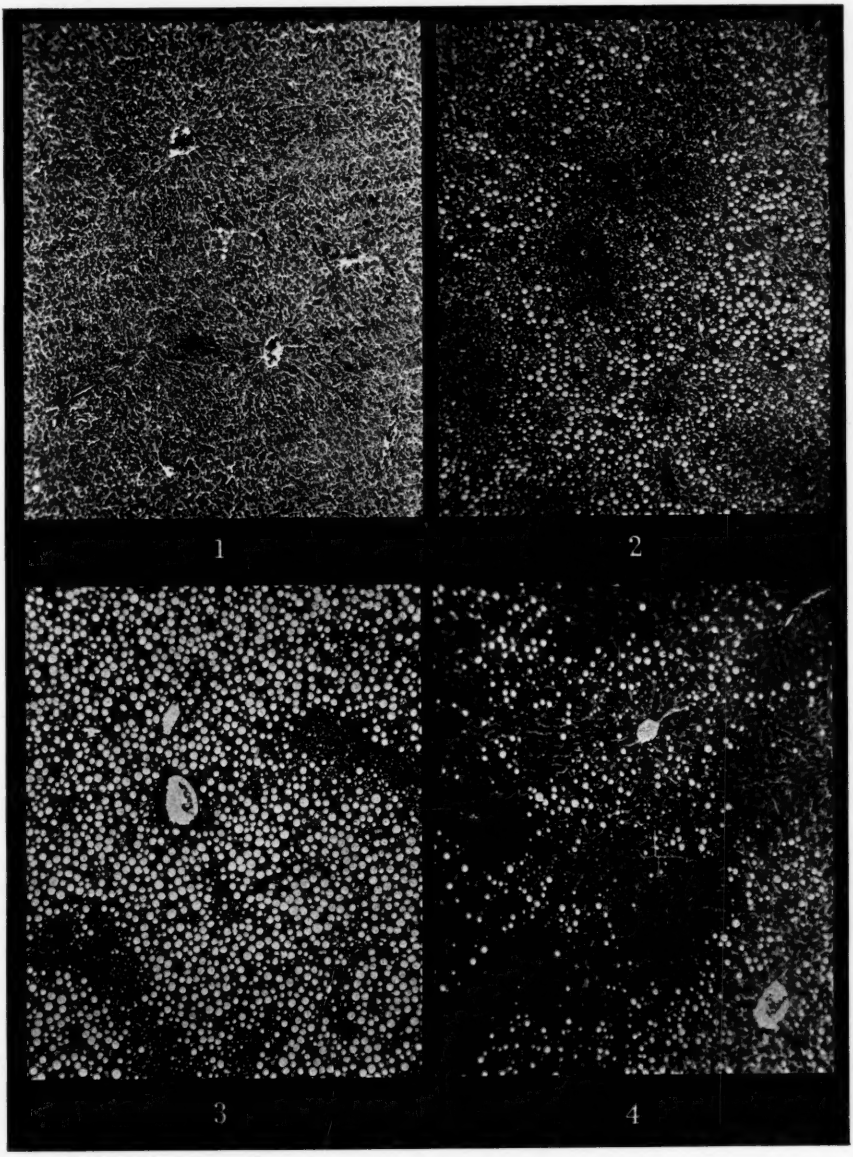
EXPLANATION OF PLATE.

FIG. 1.—Section of the liver of rat 6 (initial weight 76 g.) maintained on a methionine-choline-free diet for 36 days. Fatty change is not prominent, though present in slight degree. The principal feature is the appearance of rarefaction of the cytoplasm of the liver cells. H.E. × 85.

FIG. 2.—Section of the liver of rat 18 (initial weight 124 g.) maintained on a methionine-choline-free diet for 14 days. There is moderate fatty infiltration of the liver, associated with a loss of cytoplasm of the liver cells. H.E. × 85.

FIG. 3.—Section of the liver of rat 73 (initial weight 188 g.) maintained on a methionine-choline-free diet for 27 days. Extreme generalised fatty infiltration of the liver is present, all areas having the appearance shown. H.E. × 85.

FIG. 4.—Section of the liver of rat 75. This animal was maintained on the same methionine-choline-free diet as rat 73 for the same period of 27 days. The diet was then supplemented with these substances for a period of 10 days before the rat was killed. Recovery of the liver and disappearance of fat was patchy, but considerable areas (about 50 per cent of the whole) showed the degree of improvement illustrated. H.E. × 85.



Sims.

line maintenance, but B.D.H. fat-free casein was used when a casein hydrolysate was employed in the methionine-choline free diets. The preparation of the methionine-free casein hydrolysate was carried out by a modification of the procedure described by Albanese (1943): 200 g. fat-free casein was autoclaved for 1½ hr. at 15 lb. with 600 ml. 3 N H_2SO_4; the brownish mixture was centrifuged and the supernatant fluid allowed to stand overnight in a large beaker with 85 ml. 100 vol. H_2O_2. The mixture was then steamed for ½ hr. (an essential step to ensure complete destruction of the methionine), cooled and neutralised by the addition of a hot paste of 280 g. barium hydroxide (hydrated). The barium sulphate was separated by centrifugation, and the supernatant concentrated to a syrup-like consistency by evaporation in a shallow basin on a boiling water bath. Although hydrolysis of the casein was far from complete during the initial acid treatment, the methionine was effectively destroyed by the subsequent treatment with hydrogen peroxide, since analysis of the final liquid mixture gave values such as the following: Nitrogen 8·8 per cent, i.e., N × 6·25 55 per cent, methionine less than 0·002 per cent, choline 0·005 per cent. The lard was Adeps Praeparatus B.P., and samples of this when assayed for choline gave values of less than 0·0005 per cent. Ordinary commercial sucrose was employed, the choline content of which was found by assay to be less than 0·002 per cent. The salt mixture used was that of Hawk and Oser (1931).

Vitamin mixtures for addition to the diet were made up in the form of water-soluble and fat-soluble groups, according to the general scheme of Cuthbertson (1943, personal communication). The amounts of the pure substances used to make up 100 ml. of the water-soluble group (sufficient for 1000 g. food) were: Thiamin hydrochloride 30 mg., riboflavine 20 mg., nicotinic acid 150 mg., pyridoxine hydrochloride 10 mg., calcium pantothenate 50 mg., para-aminobenzoic acid 100 mg., inositol 250 mg., biotin 0·2 mg., folic acid 0·2 mg., choline chloride 1000 mg. (omitted in the choline-free diets), alcohol 10 ml., water to 100 ml.

For the fat-soluble vitamins DL-α-tocopherol 40 mg., vitamin A 2000 I.U., vitamin D 3000 I.U., vitamin K 1·0 mg. were dissolved in each 10 g. of Arachis oil, 150 mg. of hydroquinone being added to prevent oxidation of the α-tocopherol, and 1 g. of this mixture was added to each 100 g. of the food. This food mixture was apparently adequate in all essentials. It supported active growth in young rats, and enabled adult female rats to bear and to suckle litters normally.

When whole casein was replaced by the methionine-free casein hydrolysate, the animals received supplements of L-cystine (1–2 per cent of the protein) and DL-tryptophan (2–3 per cent of the protein) while the controls were given an additional supplement of DL-methionine (3–10 per cent of the protein in different experiments). The rate of growth shown by the control animals was inferior to that found when whole casein was the source of protein, partly owing to the fact that the hydrolysate was rather distasteful to the animals, thus leading to a reduction in the total food intake. The results obtained in many experiments showed however that the control diet had no important deficiency, as determined by growth response and by the absence of significant histological changes of the tissues examined.

RESULTS.

Throughout the whole series of experiments (which involved the examination of approximately 70 animals) the same general features were shown by rats fed

the above diet substantially free from methionine and choline. They remained apparently well, were normal in appearance and quite vigorous, but suffered a steady loss of weight associated with considerable muscular wasting, and at the end of the experiment showed varying degrees of fatty infiltration of the liver, and a reduction of the plasma protein concentration. Histological examination of liver, kidneys, spleen, lungs, heart muscle, skeletal muscle, gastric and intestinal mucosa, adrenals and bone marrow was carried out, but significant lesions were found only in the liver and in the mammary gland of the pregnant animal. The degree of fatty change present in the liver depended principally on the age of the rat and the amount of food consumed per day. In the very young and rapidly growing animals which disliked the casein hydrolysate very much and ate only small quantities of the food, fatty change was not at all a conspicuous feature, and the liver cells in these animals showed the appearance of rarefaction of cytoplasm associated characteristically with generalised protein depletion (Kosterlitz, 1947) (Fig. 1). In other cases, moderate to severe fatty change was associated with this " rarefaction " (Fig. 2). In the case of the mature adult rats, they overcame their initial objection to the food, ate quite considerable amounts per day and developed very fatty livers (Fig. 3). This result agrees with the findings of Best, Hartroft, Lucas and Ridout (1949), who showed the pronounced effect of high caloric intake on the development of fatty liver in choline-deficient rats.

Analysis of the urine for methionine and choline showed that within a day or two the excretion of methionine fell to negligible values (see Figs. 6 and 8). The choline excretion, although reduced, continued at an appreciable figure throughout the experimental period ; it remained at a higher level for young rats than for adults. Similar findings have been reported for choline by Borglin (1947). This rapid fall of the methionine excretion to negligible levels in the presence of a specific dietary deficiency of methionine is to be contrasted with the more gradual and less complete reduction of the excretion of this amino-acid when the intake of whole protein is reduced to low levels (Fig. 5). The persistence of the

TABLE I.—*Total Methionine and Choline of the Intestinal Contents of Rats.*

Rat.	Diet.	Period of experiment (days).	Total choline of upper bowel (μg.).	Total choline of lower bowel (appendix and colon) (μg.).	L-Methionine of upper bowel (μg.).	L-Methionine of lower bowel (μg.).
46	Methionine- and choline-free	7	350	14	640	620
47		7	76	17	190	610
70		27	570	120	250	620
73		27	610	130	300	420
74		27	520	130	300	540
48	Casein hydrolysate supplemented with methionine and choline	7	270	52	240	880
49		7	450	75	350	670
50	Whole casein mixture free from choline	7	132	31	280	2470
51		7	256	29	1240	2780
52	Rat cake diet	7	340	59	1010	1860
53	„ „	7	311	33	860	1200

excretion of choline in absence of any appreciable intake of this substance was an unexpected result, and gave rise to the suspicion that there might be available to the rat some source of choline other than the diet, such as a synthesis by the intestinal flora. Some experiments were undertaken to investigate this. Table I shows an analysis for choline and methionine of the total intestinal contents of a number of experimental and control animals.

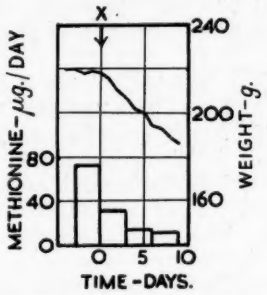


FIG. 5.—Mean values for the excretion of L-methionine during protein depletion. After 10 days on the basal diet containing 15 per cent casein the protein was replaced (at x) by sucrose.

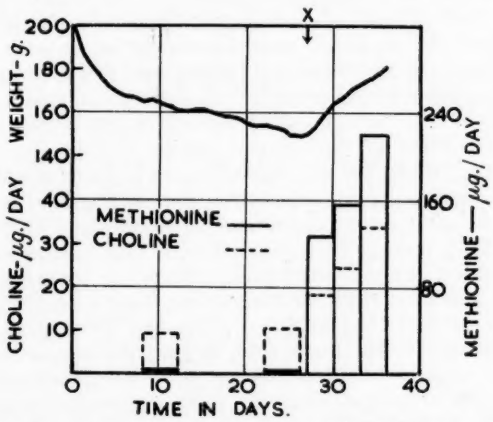


FIG. 6.—Excretion of L-methionine and choline during recovery from a methionine-choline depletion. After 27 days on a diet free from these substances, the same diet was supplemented (at x) with 0·64 per cent of DL-methionine and 0·1 per cent of choline.

It is seen that appreciable amounts of these substances are present within the lumen of the bowel of each of the deficient animals; but while the distribution of methionine is consistent with bacterial synthesis, that of choline is not, since by far the greater amount is to be found in the jejunum and ileum. Further experiments with cultures of an indole-positive strain of *Bact. coli* showed that this organism, grown on a simple inorganic salt medium with glucose (Zinsser, 1948), is capable of synthesising methionine, but not choline, and does not destroy

or take up choline when this is added to the medium. The choline of the bowel contents therefore represents most probably an excretion of phospholipid in bile, and by the mucosa, perhaps, in a similar way to the known excretion of neutral fat. The choline of this excreted phospholipid appears to be almost completely re-absorbed. The methionine synthesised by bacterial action would provide only a very small proportion of the daily requirements of the animal; the total amount present is only of the order of a few mg., and it is mostly within the bodies of the intestinal bacteria. Moreover, methionine liberated by the death and autolysis of these bacteria would be shared by competition between the bacteria and the host.

Analysis of the blood of the depleted animals showed no significant reduction in the free methionine of the plasma, although it is to be noted that Henderson, Schurr and Elvehjem (1949) found a decline in the plasma concentration of most of the free amino-acids in nitrogen-depleted rats. Neither was there a significant reduction in the total choline content of the plasma. The free choline, which forms only approximately 1 per cent of the total (Entenman and Chaikoff, 1945), was not determined. The methionine content of the plasma proteins was the same in depleted and control animals, and analysis of liver, kidney, heart muscle and skeletal muscle of the depleted animals showed no detectable reduction in the methionine content of the tissue proteins, even when the degree of depletion was extreme as measured by the extent of the liver damage. Tables II and III show a number of results obtained from two such experiments.

It was clear from these depletion experiments that no reliable information relating to the state of depletion or otherwise of an animal was likely to be obtained from analysis of the blood and tissues, and it was not at all certain that investigation of the urine would provide a more accurate gauge of the nutritional state, since the level of excretion of methionine in the urine seemed to be very largely a direct reflection of the dietary intake, and it was possible that the diminished excretion of choline was due almost wholly to a similar alteration of the diet. To attempt to decide whether a depleted animal would excrete less methionine and choline than a normal animal maintained on the same diet, six female rats, all litter-mates, were divided into two groups of three. All six were severely depleted of both methionine and choline by being maintained on a diet substantially free from these substances for three weeks. The three animals in one of the groups were then killed to prove the severity of the depletion by histological examination of the livers (Fig. 3), and the diet of the three survivors was supplemented with methionine (6·4 mg./g. of food) and choline (1 mg./g. of food). A rapid gain of weight resulted, and considerable recovery of the liver occurred during the 10 days which elapsed before the animals were killed (Fig. 4). Fig. 6 shows the mean weight curves and excretion values for choline and methionine for these animals, and it is seen that in the most severely depleted state the three animals of the second group excreted less of both choline and methionine than they did later, when a considerable degree of recovery had occurred. Although therefore the level of excretion of these substances depends very largely on the diet, it is determined to some extent by the degree of depletion of the animals in the presence of the same dietary intake.

The requirements of the pregnant animal for choline and methionine were studied in two experiments. In the first, two groups of three female rats, litter-mates, were maintained on the same diet containing 15 per cent whole casein

TABLE II.—*Methionine and Choline Content (g. per cent) of Plasma and Kidney of Young Female Rats.*

Diet.	Rat.	Free methionine in plasma (g. per cent).	Total nitrogen (g. per cent).		Total methionine (g. per cent).		Methionine × 100 / Total N.		Total choline (g. per cent).	
			Plasma.	Kidney.	Plasma.	Kidney.	Plasma.	Kidney.	Plasma.	Kidney.
Methionine- and choline-free	17	0·00055	0·85	3·03	0·078	0·42	9·1	13·7	0·014	0·26
	18	0·00065	0·86	2·94	0·080	0·39	9·3	13·4	0·017	0·23
	19	0·00049	0·66	2·92	0·072	0·39	10·8	13·5	0·010	0·24
Above diet supplemented with methionine and choline	20	0·00063	0·93	2·82	0·097	0·41	10·5	14·5	0·019	0·22
	21	0·00062	0·88	2·95	0·090	0·46	10·1	15·5	0·018	0·29
	22	0·00058	0·78	3·15	0·076	0·42	9·6	13·3	0·015	0·27

The duration of the experiment was 7 days. Figures for plasma are expressed as g. per 100 ml., while those for kidney are g. per cent of fresh tissue.

TABLE III.—*Analysis of Plasma and Tissues of Pregnant Rats and of the Tissues of the Litters.*

Estimation.	Rat.	Maternal.					Foetal.		
		Liver.	Kidney.	Heart muscle.	Skeletal muscle.	Plasma.	Liver.	Kidney.	Heart muscle.
Per cent moisture	11	51·7	77·0	78·6	76·5				
	12	54·5	77·2	77·1	77·0				
	13	56·5	78·0	78·4	80·0				
	14	71·8	77·2	72·5	74·9				
	15	70·6	75·5	76·3	74·7				
	16	69·8	75·0	77·0	74·8				
Methionine (g. per cent)	11	0·35	0·43	0·57	0·62	0·090	—	0·098	0·16
	12	0·44	0·42	0·44	0·69	0·102	0·28	—	—
	13	0·36	0·45	0·50	0·55	0·068	0·19	0·150	0·15
	14	0·56	0·42	0·48	0·70	0·110	0·32	0·130	0·18
	15	0·57	0·49	0·44	0·61	0·124	—	0·090	0·22
	16	0·55	0·48	0·55	0·68	0·098	—	—	—
Total N (g. per cent)	11	2·6	3·0	3·1	3·6	0·78	—	1·6	2·0
	12	2·9	2·9	3·1	3·9	0·90	2·8	2·5	2·5
	13	2·4	3·0	3·2	3·2	0·61	1·8	1·7	1·9
	14	3·1	2·7	3·3	3·6	0·91	2·8	1·9	1·7
	15	3·8	3·1	3·5	3·6	1·02	—	2·1	2·4
	16	3·6	3·2	3·3	3·6	0·96	—	—	—
Methionine × 100 / Total N	11	13	14	18	17	12	—	6·1	8·1
	12	15	15	14	18	11	10	—	—
	13	15	15	16	17	11	11	8·4	8·0
	14	18	16	15	19	12	11	6·8	10·6
	15	15	16	13	17	12	—	4·4	9·3
	16	15	14	17	19	10	—	—	—
Choline (g. per cent)	11	0·29	0·27	0·23	0·13	0·023	—	0·16	0·20
	12	0·40	0·28	0·29	0·13	0·025	0·19	0·20	0·20
	13	0·26	0·25	0·22	0·13	0·028	0·08	0·15	0·18
	14	0·43	0·25	0·25	0·12	0·029	0·24	0·15	0·18
	15	0·62	0·37	0·34	0·13	0·037	—	0·17	0·22
	16	0·42	0·26	0·25	0·14	0·024	—	—	—

Rats 11, 12, 13 were depleted animals, and 14, 15, 16 had the same diet supplemented with 1 per cent of DL-methionine and 0·1 per cent choline. Figures for plasma are expressed as g. per 100 ml., those for the various tissues are in g. per 100 g. of fresh tissue.

and supplemented with choline. One group was mated and the excretion of these substances was followed and compared with that of the control group during the ensuing three weeks. Fig. 7 shows the mean weight curve and the excretion of choline and methionine for the pregnant and control groups; and it can be

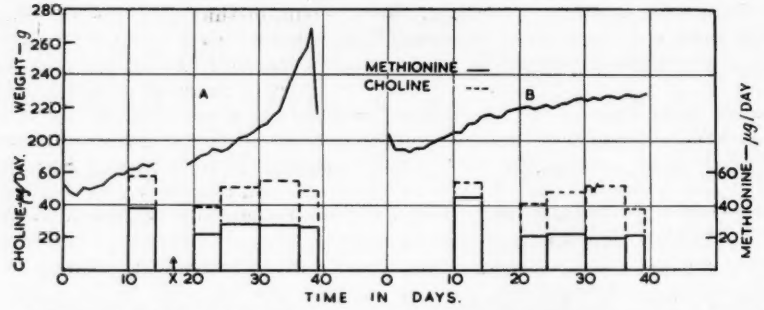


FIG. 7.—Excretion of L-methionine and choline by pregnant and non-pregnant rats (fed the basal diet containing 15 per cent casein). Sperms were present in vaginal smears of group A (pregnant animals) at *x*. Group B are non-pregnant.

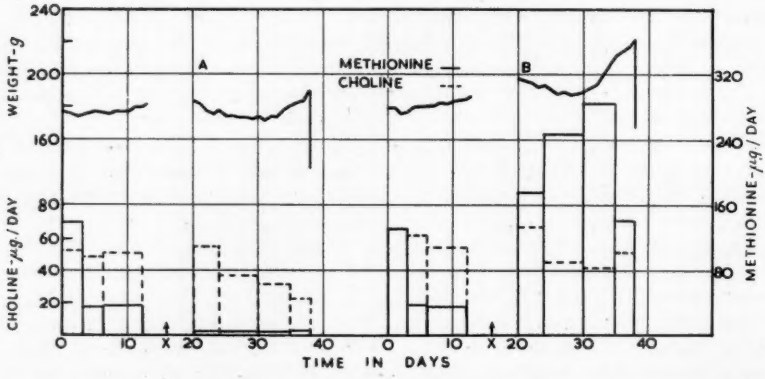


FIG. 8.—Excretion of L-methionine and choline in pregnant depleted rats. At *x* (vaginal smears contained sperms) group A animals were placed on a methionine-choline free diet, and group B on the same diet supplemented with 1 per cent of DL-methionine and 0·1 per cent of choline.

seen that there was no immediate change in the excretion of these substances when the animals became pregnant, neither was there any appreciable falling off in the level of excretion during the latter part of pregnancy. In none of the livers was there any fatty change. This result suggests that in pregnancy in the rat there is no unusually heavy demand for choline and methionine speci-

fically, although the plasma protein levels of the pregnant animals were lower than for the non-pregnant group, and there was some histological evidence of mobilisation of liver protein in the pregnant rats.

The second experiment was a comparison of two groups of pregnant animals maintained on a diet containing 10 per cent casein hydrolysate. One group was fed the diet free from methionine and choline, the other having the same diet liberally supplemented with methionine (DL-methionine = 10 per cent of the protein), and containing the usual 1 mg. choline per g. food. The animals were placed on the experimental diet as soon as mating had occurred and sperms were seen in a vaginal smear. One of the control animals failed to become pregnant, but all the other rats carried their litters to term. The control animals had normal litters, and suckled normally, but the offspring of the depleted rats were weakly and in each case the mothers began to eat the young at birth. The most probable reason for this was a failure of lactation, since sections of the mammary tissue of the depleted animals showed a striking absence of secretory activity compared with that of the controls.

Analysis of the tissues of the mothers and of the litters of the two groups of rats showed no significant difference between the depleted and the control animals except for the maternal livers in which the gross fatty change of the depleted animals gave lower results due to the presence of the neutral fat (Table III). Histological sections of the livers of the depleted offspring showed well-marked changes of the liver cells. Appearance of rarefaction of the cytoplasm was prominent, and there was a much greater irregularity of cell and nuclear size than in offspring of the controls. There was no fatty change in the livers of the litters of the depleted animals.

Fig. 8 shows the average weight changes and the methionine and choline excretion during pregnancy for the depleted and control groups of animals. It is seen that there is a considerable fall in the choline excretion of the depleted animals, but this was probably no more than would have occurred in the absence of pregnancy. The blood of the two groups of animals showed no significant differences except for the fall in the plasma proteins of the depleted animals (a mean of 4·8 g. per cent compared with 6·0 g. per cent for the controls).

DISCUSSION.

The experiments described above provide a further demonstration of the ability of the body to maintain in a constant state the composition of the various tissues even in the face of an extreme deficiency. Here, the animals were maintained on diets which contained no more than minute traces of methionine and choline ; yet except for the progressive loss of weight they remained apparently well, and, except for a fall in the plasma proteins, the blood and tissue levels of these substances remained normal within the limits of the experimental error of the method of assay. Since both choline and methionine are subject to a certain rate of loss by degradation, this maintenance of a uniform cell environment could have been achieved only at the cost of the mobilisation of certain of the tissues of the body. At first, as in the experiments of Kosterlitz (1947), the protein of the liver cells was used for this purpose, and there was clear histological evidence of this, but later when this readily available source was seriously depleted, the generalised muscular wasting observed indicated a widespread

mobilisation of tissue, affecting muscle more particularly. The rate of loss of weight of the depleted animals if regarded as due to muscular wasting would supply the rats with sufficient choline and methionine for the very modified bodily requirements appropriate to their condition, and in pregnancy was sufficient to account for the methionine and choline content of the litter, as well as the daily requirements of the mother. Those changes in the methionine content of plasma and tissue proteins which were found were those associated with growth, the immature animal having a lower protein content of the tissues and a proportionately lower methionine content of this tissue protein.

The fact that the methionine excretion of the rat is largely determined by the dietary intake of this substance is in agreement with the experimental findings of Sauberlich and Baumann (1946), and similar to those of Schweigert, Sauberlich, Elvehjem and Baumann (1946), who studied tryptophan.

Similar conclusions for man are suggested by the experiments of Eckhardt and Davidson (1949) on a fasting subject, those of Dunn, Camien, Shankman and Block (1947) on a subject changed from a normal diet to Army K ration, and the results obtained by Frankl and Dunn (1947) in an investigation of the excretion of tryptophan.

Although excretion studies of the pregnant rat have offered little evidence for the existence of a more than usual demand for labile methyl groups in this condition, it is clear from the gross and microscopic examination of mammary tissue that choline or methionine or both are necessary for normal lactation. Jacobi, Baumann and Meek (1941) investigating the effect of choline deficiency on reproduction, reared a number of rats, using as food a choline-free diet with 25 per cent casein as the source of protein. They found that 14 out of 15 such rats grew to maturity and gave birth to litters, although in the second generation the rate of growth was somewhat less in the deficient animals. On the other hand, Sure (1940) found that the litters of the choline-deficient rat developed paralytic symptoms about the 13th day, and in the absence of a choline supplement died between the 15th and 18th days, from which he concluded that choline was an essential substance for normal lactation. It is not at the moment clear how these two results may be reconciled; Sure's diet may have been deficient in other essential constituents besides choline, but it would appear at all events that the actual process of secretion of milk is comparatively normal in animals receiving methionine but no choline, whatever the quality of this milk may be. In the experiments here described there was very little secretion of milk in the depleted animals, and sections of the mammary glands show a striking absence of secretory activity compared with the controls. It would therefore seem probable that methionine in adequate quantities is essential for normal lactation. This is consistent with observations made on humans under famine conditions. Under such circumstances although pregnancy may proceed until near term with no apparent abnormality, lactation is in most cases considerably reduced, both the quantity of milk available and the duration of lactation suffering as a result of the deprivation of high grade protein.

A study of the histological appearances of the liver cells obviously provides a sensitive index of the state of nutrition of the animal. The observed effects of methionine depletion would suggest that a shortage of any essential amino-acid will lead to mobilisation of the cytoplasm of the liver cells such as occurs in total protein deprivation (Kosterlitz, 1947).

SUMMARY.

Histological and biochemical features of a combined methionine-choline deficiency were studied in groups of rats maintained on diets composed of purified ingredients with casein or casein hydrolysate as the source of protein.

Significant pathological lesions were found only in the liver, and in the mammary tissue of pregnant animals. The liver lesions consisted of a mobilisation of the cytoplasm of the liver cells accompanied by fatty infiltration the degree of which was dependent on the total carbohydrate and fat intake. There was an absence of secretory activity of the mammary glands of the depleted pregnant rats.

There were no significant changes in the blood or tissue concentrations of choline and methionine in the depleted animals, and no detectable changes in the methionine content of the plasma and tissue proteins. There was, however, a fall in the plasma protein level as a result of the deficiency.

The excretion of methionine largely reflected the dietary intake of this amino-acid, but on a given diet a severely depleted animal excreted less than the normal rat. Although the excretion of choline followed the intake less closely (tending towards a constant excretion in the young rat), there was, in the adult animal on a given diet, a lower level of excretion in the presence of a gross depletion of this substance.

No evidence was found of an unusually heavy demand for methionine and choline (labile methyl groups) in pregnancy, although it appears that the health of the liver cells (maternal and foetal) and lactation in the rat are impaired in the presence of a specific methionine-choline deficiency.

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THE COMPARATIVE EFFECT OF ALPHA-TOCOPHEROL AND ITS ANALOGUES ON MASSIVE HEPATIC NECROSIS IN RATS ON A PROTEIN DEFICIENT DIET.

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IT has been shown by Glynn, Himsworth and Neuberger (1945) and György (1947) that the massive hepatic necrosis produced in rats by a protein-deficient diet could be prevented by supplements of either cystine or tocopherol. The present investigation was undertaken to determine the amount of DL-α-tocopherol acetate required to give this protection to rats on a low-protein diet, and to compare the protective effect on the liver of α-tocopherol with that of its analogues.

MATERIALS AND METHODS.

Diet.

A diet which had proved to be satisfactory in the production of hepatic necrosis was one suggested to us by Gillman (1949). It consists of 90 per cent potato starch and 10 per cent food yeast. Unfortunately potato starch became unobtainable so corn starch was substituted, and this proved to be equally satisfactory. This corn starch and food yeast diet was prepared as follows: 90 g. corn starch was made into a smooth paste with 100 ml. water and added to 500 ml. boiling water, the mixture being stirred thoroughly and boiled for a further 2 min. When cool, 10 g. food yeast in 20 ml. water was added and stirred in thoroughly. This diet, without any supplement, was fed to rats *ad lib.* In addition they were allowed tap-water.

Animals.—Albino rats weighing between 30 and 66 g. were used. The mothers of the litters had been on a normal stock cubed diet consisting of yellow maize meal 48 per cent, peanut meal 10 per cent, meat meal 15 per cent, fish meal 2·5 per cent, oatmeal 15 per cent, barley 7·5 per cent, lucerne 1 per cent, salts 0·5 per cent, and limestone powder 0·5 per cent, with a supplement of green vegetables given twice a week. The infant rats nibbled at this diet until they were separated for the experiment.

Tocopherol.—In a pilot experiment it was found that weekly doses of 2 mg. DL-α-tocopherol acetate completely protected rats fed on corn starch and food yeast against massive hepatic necrosis. Smaller doses were therefore used in the experiments to be reported. In all the experiments the DL-α-tocopherol acetate, or the particular analogue being tested, was dissolved in arachis oil and the solution was given by oesophageal tube. Varying concentrations were prepared so that the dose of tocopherol was always contained in 0·3 ml. solution.

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RESULTS.

The minimal dose of DL-α-*tocopherol acetate which will prevent massive hepatic necrosis in rats on a protein-deficient diet.*

Experiment 1.—Sixty-four rats aged 3 to 4 weeks and weighing between 31 and 56 g. were used. They were housed in separate cages, were weighed weekly, and all were given the same basic diet of corn starch and food yeast. The rats were divided into 6 groups. Groups I and II, each consisting of 12 rats, were used as controls. Group I was fed on the basic diet alone. Group II received 0·3 ml. arachis oil weekly, to exclude the possibility that the arachis oil itself contained any protective substance. Groups III, IV, V and VI, each consisting of 10 rats, received respectively 0·1 mg., 0·2 mg., 0·4 mg. and 0·8 mg. DL-α-tocopherol acetate in arachis oil weekly.

Results.—All the 24 control rats (Groups I and II) developed hepatic lesions. In Group I, fed on the basic diet alone, massive hepatic necrosis was present in 10 rats and the remaining 2 had healed hepatic lesions. In Group II, which received arachis oil in addition to the basic diet, massive hepatic necrosis was present in all. Death from hepatic necrosis in these groups occurred as early as the 26th day after commencement of the feeding and by the 59th day all the rats were dead.

Of the rats in Group III, each of which had received 0·1 mg. DL-α-tocopherol acetate weekly, 7 died between the 28th and the 55th day as a result of massive hepatic necrosis. One animal was killed accidentally. The remaining 2 showed no evidence of hepatic damage ; one of these died on the 75th day and the other was killed on the 95th day.

Of the 10 animals in Group IV, each of which had received 0·2 mg. DL-α-tocopherol acetate weekly, 6 had recent or old hepatic lesions. In 2 of these the lesion was massive hepatic necrosis only, in 2 there was post-necrotic scarring only, whilst the other 2 showed both types of lesions. Five of these 6 animals died between the 52nd and 76th day and the other was killed on the 95th day. In the remaining 4 rats of this group no hepatic lesion was found ; 3 of these died from unexplained causes between the 35th and 83rd day, and the fourth was killed on the 95th day.

Of the rats in Group V, each of which had received 0·4 mg. DL-α-tocopherol acetate weekly, only one had a liver lesion (massive hepatic necrosis). This animal died on the 82nd day of the experiment. Of the remaining 9, 1 died on the 41st day, and the others survived as long as 75 to 95 days after commencement of the experiment.

The animals in Group VI, each of which had received 0·8 mg. DL-α-tocopherol acetate weekly, gave results similar to those found in Group V. Only one rat developed massive necrosis, and its death occurred on the 87th day of the experiment.

In all groups the animals showed an initial gain in weight of from 6 to 22 g., which in the majority of cases was wholly or partly lost towards the end of the experiment. These results are summarised in Table I.

It was concluded that 0·4 mg. DL-α-tocopherol acetate per week is the minimum dose which will give a substantial protection to rats on a diet of corn starch and food yeast from developing hepatic necrosis. Furthermore, it appeared that the period of survival of rats that developed liver lesions whilst receiving 0·2 mg. DL-α-tocopherol acetate was longer than in control animals.

TABLE I.—*Summary and Statistical Analysis of the Results of Exp.* 1.

Group.	Dose of Tocopherol (mg./week).	Number of rats.	% with liver lesions.	Difference from % in Group I.	2 × S.E. of difference.	Statistical significance of difference.
I	0 (Control)	12	100	—	—	—
II	0 (Arachis oil)	12	100	—	—	—
III	0·1	9	78	22	27·6	Not significant
IV	0·2	10	60	40	31	Significant
V	0·4	10	10	90	34	,,
VI	0·8	10	10	90	42	,,

The effectiveness of some analogues of DL-α-*tocopherol in protecting rats on a protein-deficient diet from massive hepatic necrosis.*

Experiment 2.—All the rats in this experiment were given the corn starch and food yeast diet as in Exp. 1. The tocopherol analogues tested were dissolved in arachis oil in exactly the same way as the α-tocopherol in Exp. 1. Each solution was administered by oesophageal tube in weekly doses of 0·3 ml., each dose containing the amount of the particular analogue equivalent on a molar basis to 0·4 mg. DL-α-tocopherol acetate. Two groups of rats were given α-tocopherol-quinone at weekly intervals, one group receiving 20 mg. per dose and the other 5 mg.

One hundred and six rats were used, divided into 8 groups. Groups I and II, each consisting of 14 animals, were controls. Group I received the basic diet alone, while Group II received 0·3 ml. arachis oil weekly in addition. Groups III, IV and V, each consisting of 15 rats, were given respectively D-δ-tocopherol, D-γ-tocopherol, and D-α-tocopherol, in addition to the basic diet. Group VI, consisting of 14 rats, received DL-α-tocopherol acetate. Groups VII and VIII, consisting of 10 and 9 rats each, received respectively 20 mg. and 5 mg. α-tocopherol-quinone weekly.

TABLE II.—*Summary and Statistical Analysis of the Results of Exp.* 2.

Group.	Tocopherol.	Number of rats.	% with liver lesions.	Comparison with group.	Difference of % from % in group compared.	2 × S.E. of difference.	Statistical significance of difference.
I	0 (Control)	14	100	—	—	—	—
II	0 (Arachis oil)	14	100	—	—	—	—
III	delta	15	100	—	—	—	—
IV	gamma	15	93	I (control)	7	13·4	Not significant
V	D-alpha	15	60	I ,,	40	31·6	Significant
				IV (gamma)	33	32·2	,,
VI	D-L-alpha	14	50	I (control)	50	32·6	,,
				IV (gamma)	43	33·6	,,
				V (D-alpha)	10	37	Not significant

Results.—All 28 rats in the control groups I and II developed massive hepatic necrosis. Death occurred in all cases between the 23rd and 65th day of the experiment. All 15 rats in Group III, receiving D-δ-tocopherol, developed hepatic lesions, there being massive necrosis in 11 and post-necrotic scarring in the remaining 4. The earliest death occurred on the 35th day and the latest on the 69th day. Fourteen of the 15 rats in Group IV, receiving D-γ-tocopherol, showed massive hepatic necrosis, and death occurred between the 25th and 79th

days. Seven of the 15 rats in Group V, receiving D-α-tocopherol, had massive necrosis, and in 2 others there was scarring with small areas of recent necrosis. The deaths of rats with hepatic lesions occurred between the 46th and 90th day. Seven of the 14 in Group VI, receiving DL-α-tocopherol, showed hepatic necrosis, and death occurred between the 55th and 85th day.

None of the rats in Groups VII and VIII, receiving respectively 20 mg. and 5 mg. α-tocopherol-quinone weekly, showed old or recent hepatic lesions. Death occurred between the 54th and 95th day in Group VII, and between the 49th and 87th day in Group VIII.

An initial gain in weight was less consistently seen in Exp. 2 than in the previous experiment. Twenty-four rats which subsequently developed massive hepatic necrosis and 9 which did not showed no increase of weight while on the corn starch and food yeast diet.

DISCUSSION.

In the first experiment 0·4 mg. of DL-α-tocopherol acetate protected 90 per cent of rats on a protein-deficient diet from massive hepatic necrosis. In the second experiment this dosage only afforded a 50 per cent protection. The reason for this is not clear. A comparison with the analogues shows that in equivalent small dosages neither γ- nor δ-tocopherol will protect rats from hepatic necrosis (β-tocopherol was not available for the test). In a subsequent experiment it was found that weekly doses of 0·8 mg. of either D-γ-, or D-δ-tocopherol failed to prevent hepatic lesions in 90 per cent of rats. The rats receiving γ-tocopherol lived for a longer period before manifesting their lesions than those receiving D-δ-tocopherol. D-α-tocopherol was almost as effective as DL-α-tocopherol in protecting against liver lesions, and statistical examination showed no significant difference between the results obtained with these two analogues.

α-Tocopherol-quinone given to pregnant mice on a normal diet is known to result in resorptive interruption of pregnancy, whereas α-tocopherol promotes a continued normal pregnancy (Woolley, 1945). Contrary to expectation α-tocopherol-quinone in doses of either 20 or 5 mg. weekly was found to give 100 per cent protection from hepatic lesions in our experiments. Our preparation of α-tocopherol-quinone contained approximately 3 per cent of α-tocopherol. Hence the rats given the larger dose of the quinone would have received more than the minimum dose of free α-tocopherol necessary to protect the liver. However, the rats given the smaller dose (5 mg.) of quinone were receiving 0·15 mg. α-tocopherol, which is less than the minimal protecting dose. Far from being an antagonist to α-tocopherol in liver necrosis α-tocopherol-quinone, itself, protects the liver.

The value of α-tocopherol in diets is obvious. Certain African tribes live on a diet consisting chiefly of soured milk and mealiemeal (maize) with very little protein. It has been shown by Emerson, Emerson and Evans (1939) and confirmed by Hove and Hove (1944) that corn (maize) contains predominantly γ-tocopherol and " perhaps a trace " of the alpha form. The above experiments show that γ-tocopherol has not the same liver protective value as the α form. When, in addition, it is considered that much of the tocopherol which is present in the germ of maize is lost in the milling process, the deficiency of tocopherol may become an important factor in the high incidence of liver disease amongst Africans.

SUMMARY.

1. 100 per cent of young rats fed a diet of 90 per cent corn starch and 10 per cent food yeast developed hepatic lesions.

2. In Exp. I the weekly administration of 0·4 mg. of DL-α-tocopherol acetate orally *prevented* liver lesions in 90 per cent of the rats and in Exp. II in 50 per cent, whilst D-α-tocopherol afforded protection in 40 per cent of rats.

3. Doses of D-γ-tocopherol equivalent on a molar basis to 0·4 mg. D-L-α-tocopherol acetate failed to protect young rats on the protein-deficient diet from liver lesions in 93 per cent of cases, and the D-δ-tocopherol failed in 100 per cent.

4. The administration of 20 mg. and 5 mg. of α-tocopherol-quinone weekly to two groups of rats on a protein deficient diet resulted in complete protection from liver lesions.

5. The importance of the above findings in relation to the high incidence of liver disease in certain African tribes whose chief diet is mealiemeal (maize) and sour milk is pointed out.

We wish to thank Distillation Products, Inc., Rochester, New York, for supplying us with D-α-, D-γ-, and D-δ-tocopherols, and Dr. Sevringhaus, Hoffmann-La Roche, Inc., for preparing the α-tocopherol-quinone.

We are especially indebted to Professor M. van den Ende and Dr. T. Mead for many helpful suggestions and criticism.

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STUDIES ON THE REPRODUCTION OF BACTERIAL VIRUSES. I. SOME OBSERVATIONS ON THE ADSORPTION AND MULTIPLICATION OF COLIPHAGES.

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AMONG the numerous investigations over the past decade of the multiplication of viruses a number have been concerned with the adsorption and subsequent elution or release of the virus from susceptible cells. The complexity of the systems involved, however, has limited studies to a few cases in which the process could be followed with relative ease. Factors concerned in the adsorption of the mumps-influenza group of viruses on erythrocytes have been studies by many workers, and extended to adsorption of influenza virus on isolated perfused ferret lung (Hirst, 1943) and of mumps and influenza on the chorio-allantois of the chick embryo (Henle and Rosenberg, 1949; Ginsberg, Goebel and Horsfall, 1948), and similar studies have been made with the pneumonia virus of mice (Horsfall and McCarty, 1947), but all these have been concerned rather with the chemical nature of the cell receptors and with virus inhibitors than with the kinetics of the process.

Similarly, in the case of bacteriophages, much of the recent work has been concerned with the effect of mutations of host and virus on adsorption and co-factor requirements, whilst the "single-step" method of studying bacteriophage multiplication (Ellis and Delbrück, 1939), which has proved so valuable in many respects, gives no information about the initial adsorption period. In essentials this method involves the mixing of bacteria and phage in nutrient medium at high concentration for a short period to ensure rapid adsorption of the virus on the host cell, and then dilution of the mixture several thousand-fold, so that any phage released will not be further adsorbed. Periodical assays, carried out on the diluted mixture, demonstrate the course of virus multiplication and release, and enable the time interval from mixing of phage and bacteria to release of phage (latent period), average yield of phage per bacterium (burst-size), and other factors to be calculated. But obviously these results are not applicable to the preliminary period of adsorption. Several workers have, however, shown that adsorption of phages on bacteria occurs at a logarithmic rate (cf. Delbrück, 1940*a*), and both the adsorption-rate constant for T4 coliphage activated by tryptophane and its rate of de-activation when the concentration of tryptophane is reduced below the minimum required for activation have been calculated (Anderson, 1949; Wollman and Stent, 1950; Stent and Wollman, 1950).

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It seemed worth while, however, to undertake further studies of the process of phage adsorption and multiplication, and at the same time to devise means whereby the phage and its host cells were present in sufficient concentration to allow the increase in phage to be followed simultaneously by plaque counts and by microchemical estimations—which the "single-step" experiment does not permit. Thus the present paper deals with the development of a method for studying the adsorption of the T series of phages on *Bact.* (*Escherichia*) *coli*, strain B, by centrifuging down the bacteria with their adsorbed phage at various time intervals and measuring the residual phage in the supernatant; and it includes also application of the method to problems connected with bacteriophage reproduction. A preliminary report of part of this work has already appeared (Dolby and Czekalowski, 1949).

MATERIALS AND METHODS.

Media.

For propagation of the phages and for experimental purposes two media have been used: (*a*) a complex medium (GTP-broth) containing 2 per cent Difco Tryptose; 0·2 per cent glucose; 0·5 per cent NcCl; and 0·01M phosphate, pH 7·2, and (*b*) a synthetic medium (S-medium) containing 6·0 g. Na_2HPO_4 anhydrous; 3·0 g. KH_2PO_4; 0·2 g. $MgSO_4$; 1·0 g. NH_4Cl; 0·5 g. NaCl; and 4·0 g. glucose per litre. The diluting fluid in the earlier experiments was 0·85 per cent saline containing 0·01M phosphate buffer, pH. 7·2, in which the phages were found to be stable for at least two hours at laboratory temperature. But, in view of the results of Adams (1949) on the stability of the T series of phages in salt solutions, 0·005M $MgCl_2$ was added to the phosphate-saline in later experiments.

Phages.

Bact. coli, strain B, and the T series of phages, for which we are indebted to Dr. W. J. Elford, F.R.S., have been used throughout this work. The wild types of the phages have been employed and no attempt made to separate the r and r^+ mutants (Hershey, 1946). Most experiments have been carried out with the T2 phage.

Propagation of the phages was carried out by growing *Bact. coli* B in the appropriate medium to about 1×10^8 organisms per ml. and adding phage to a final concentration of 1×10^9 lytic particles per ml. The mixtures were incubated at 37° for 4–6 hours and then stored at 4° for 7–14 days before filtration through sintered-glass filters, porosity 5-on-3, to remove residual bacteria. In our experience, unlike that of Delbrück (1940*b*), this filtration caused no loss of phage, titres of the filtrates being usually 5×10^9 to 5×10^{10} lytic particles per ml. The titre of the T2 phage generally doubled over a period of a fortnight after filtration and then remained stable for at least six months.

Phage counts.

These were carried out by the plaque-counting method of Hershey, Kalmanson and Bronfenbrenner (1943). In early experiments titration was done on GTP-broth solidified with 1·2–1·5 per cent agar and with 3 ml. of GTP-broth solidified with 0·7 per cent agar for the supernatant layer. Later, Difco broth agar was used for the base and meat-extract-peptone agar for the upper layer.

Bacterial counts.

Enumeration of the *Bact. coli* B was by viable count carried out on dilutions of the experimental culture made two minutes before the addition of the phage.

RESULTS.

Preliminary experiments.

Dilutions of the T series of phages in phosphate-saline were found, as stated above (under "Materials and Methods"), to be stable for at least two hours at room temperature.

Preliminary studies showed that the process of adsorption and multiplication of the phage could be followed satisfactorily by the assay of the supernatant after centrifuging, and further experiments were undertaken to determine the

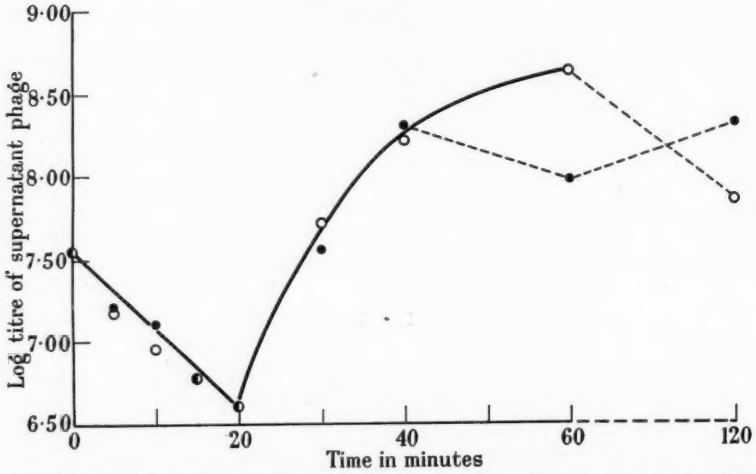

FIG. 1.—Multiplication of T2 phage on washed *Bact. coli* B in saline and in saline-phosphate. Initial titre of T2 phage, $3{\cdot}65 \times 10^7$ lytic particles per ml.
●——●, Saline (pH 7·0) suspension of cells, $7{\cdot}3 \times 10^7$ viable organisms per ml.
○——○, Saline-phosphate (0·01M phosphate, pH 7·0) suspension of cells, $6{\cdot}5 \times 10^7$ viable organisms per ml.

effect on the phage-bacteria system of dilution in saline and in phosphate-saline. A four-hour aerated culture of *Bact. coli* B in S-medium was centrifuged, washed twice in saline (pH 7·0) and re-suspended in saline and in phosphate-saline at 37°. Phage T2 in S-medium was added to each suspension to give single infection, and the course of the adsorption process followed by dilution of aliquots of the mixtures into phosphate-saline, centrifuging and assaying the phage in the supernatant. The results (Fig. 1) show that an increase in titre of phage from log 7·5606 to log 8·3053 (about 6-fold) in saline and from log 7·5606 to log 8·6877 (13-fold) in phosphate-saline had occurred after 40 and 60 min. at 37° respectively. This may be referable to the presence of metabolites inside the bacteria which can be used for synthesis of phage even when they are not replaced by nutrients

from the medium, although the extent of synthesis is naturally limited. To eliminate this source of error dilution tubes in all future experiments were kept in an ice-water bath, at which temperature this increase in titre was negligible.

The slow rise in titre of T2 phage after preparation seems to indicate a process of disaggregation—a possibility supported by certain other facts (Baylor and Clark, 1947; Doermann, 1948). If phage is released from infected cells in an

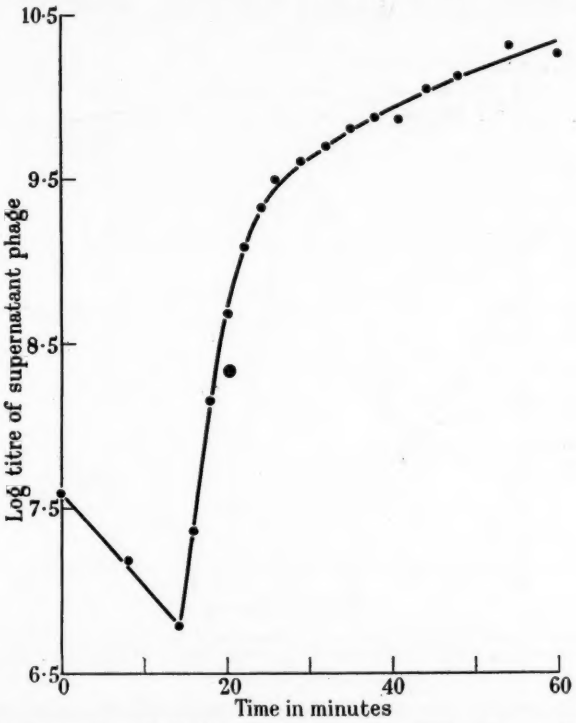

FIG. 2.—Adsorption and multiplication of T2 phage in GTP-medium. *Bact. coli* B, $8{\cdot}6 \times 10^7$ viable organisms per ml. Initial titre of T2 phage, $3{\cdot}90 \times 10^7$ lytic particles per ml.

aggregated form the value of results is greatly reduced. An attempt was therefore made, by parallel dilution of a coli-phage mixture at intervals into normal saline-phosphate and into 10 per cent saline-phosphate, to discover whether such aggregation does in fact occur, but with entirely negative results.

Final method.

The method finally adopted was as follows: *Bact. coli* B was grown to the appropriate population (usually $5–10 \times 10^7$ per ml.), phage added to give infection of each bacterial cell by no more than one phage particle, and the

subsequent titre of phage in the supernatant followed at intervals by diluting aliquots of the mixture 200-fold into saline-phosphate at 4°, centrifuging the diluted mixture at 3500 r.p.m. for 15 min. in an angle centrifuge and assaying the supernatant. Throughout the paper supernatant phage titres in the figures are those of the original mixtures before dilution.

Examples of the type of curve obtained in a large number of experiments of this kind are given in Fig. 2 and 3 for the multiplication of T2 phage on *Bact. coli* B in GTP-medium and in S-medium respectively. In both media adsorption

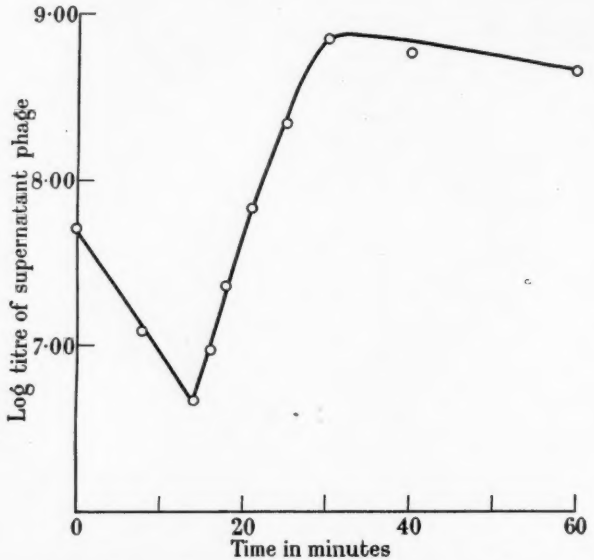

FIG. 3.—Adsorption and multiplication of T2 phage in S-medium. *Bact. coli* B, $4{\cdot}4 \times 10^7$ viable organisms per ml. Initial titre of T2 phage, $5{\cdot}05 \times 10^7$ lytic particles per ml.

proceeds logarithmically for 13–14 min.; in these particular experiments the adsorption rate constants (defined as k in the equation $-\frac{d\phi}{dt} = k\phi B$, where ϕ = concentration of virus remaining unattached to cells at time t, and B = concentration of bacteria) are 155×10^{-11} ml./min. in GTP-medium and 380×10^{-11} ml./min. in S-medium, results which are of the same order as those obtained by other workers (Anderson, 1949; Wollman and Stent, 1950; Puck, Garen and Cline, 1951). After 14 min. there is a sudden reversal of the curve—presumably due to lysis of those bacteria infected earliest—and the titre of phage in the supernatant increases at a logarithmic rate during the early stages. The curve then levels off and, as other experiments show (Fig. 4 and 7), the titre decreases slightly, owing to readsorption, before attaining its final level. This pattern has been constant throughout our work, except for anomalous results in a few early experiments when adsorption appeared to be complete in

8 min. and release of the virus produced by the bacteria occurred in phases interrupted by periods of re-adsorption. Neither exact repetition nor alteration of conditions has enabled us to repeat these results and they remain unexplained.

A comparison of this type of curve with that obtained in " single-step " experiments immediately reveals a considerable difference. The two types of

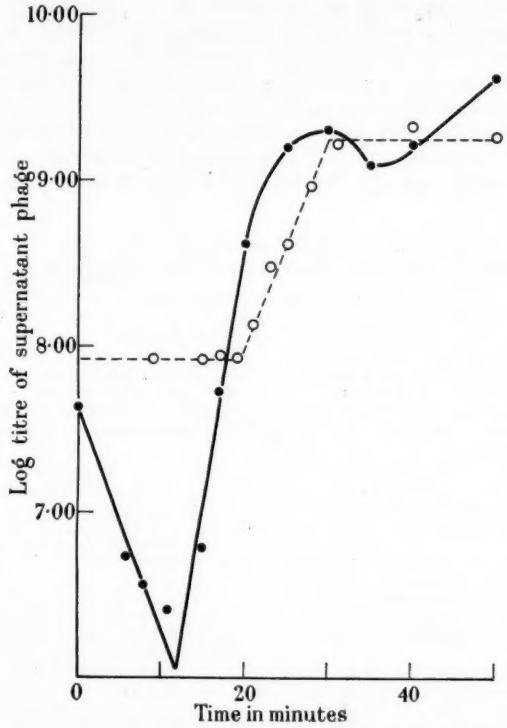

FIG. 4.—Adsorption and multiplication of T2 phage followed simultaneously by " single-step " and " supernatant " methods. For experimental details see Table I.

experiment are not strictly comparable in the adsorption stage as the conditions of adsorption and dilution are different, but it might be expected that once adsorption of a phage particle to a bacterium had occurred in a given medium, the length of time required by it for multiplication and for lysis of the bacterial cell would be constant. But, in fact, whereas these experiments show lysis beginning at 13–14 min. with phage T2, in " single-step " experiments it does not begin until 19–21 min. A direct comparison of the two methods therefore seemed essential and was made in a number of experiments ; the details of one are shown in Table I and the results illustrated in Fig. 4, in which the titres in the " single-step " experiment have been converted, for purposes of comparison, into the titres which would have been found in the original coli-phage mixture

before dilution. It will be seen that this experiment not only confirms previous results, but shows thatb rise in titre in the " single-step " experiment corresponds in time to the upper part of the curve produced by the " supernatant " method. Moreover, the final titre in the " single-step " method corresponds to the maximum titre of the " supernatant " method before re-adsorption and further multiplication occur. Another point of interest is the discrepancy between the initial titre of phage, as shown by a preliminary titration of the phage before its addition to the *Bact. coli* culture, and the level of phage during the latent period in the " single-step " experiment; the former is shown in Fig. 4 by the titre at zero time for the " supernatant " method curve, and the latter by the level initial portion of the " single-step " curve. The titres would be expected to be the same but, in point of fact, that of the " single-step " experiment is twice that of the " supernatant " experiment. Similar results have been obtained repeatedly, but an explanation is not so far forthcoming.

TABLE I.—*Experimental Procedure for the Comparison of the " Single-step " and and " Supernatant " Methods of Following the Multiplication of T2 Phage.*

Time (min.).		
−150	.	0·05 ml. of an 18-hr. GTP-broth culture of *Bact. coli* B inoculated into 20 ml. GTP-broth and incubated at 37°.
−10	.	Assay of diluted stock T2 phage. Titre $8·9 \times 10^8$ particles/ml.
−2	.	Assay of *Bact. coli* B culture. Titre $1·13 \times 10^8$ organisms/ml.
0	.	1·0 ml. diluted stock T2 phage added to *Bact. coli* B culture (Tube B/T2) which contained: *Bact. coli* B, $10·8 \times 10^7$ organisms/ml. T2 phage, $4·2 \times 10^7$ lytic particles/ml.
5		" Single-step " technique. — " Supernatant " technique.
		0·05 ml. Tube B/T2 added to 5 ml. GTP-broth. From this tube: — At intervals 0·1 ml. from Tube B/T2 diluted into 20 ml. phosphate-saline in ice-water bath. Aliquot spun 15 min. at 3500 r.p.m.
		0·05 ml. added to 25 ml. GTP-broth at 37°. — 0·2 ml. from Tube I added to 20 ml. GTP-broth at 37°.
		Growth Tube I. — *Growth Tube II.*
		0·5 ml. samples from Tubes I and II taken at intervals and assayed without 5 min. adsorption on *Bact. coli* B. — Sampled at 6, 8, 15, 17, 20, 25, 30, 35 min. Supernatant from centrifugation diluted 1/200 in phosphate-saline and 0·5 ml. assayed with 5 min. adsorption on *Bact. coli* B.
		Sampled at 8, 15, 17, 19 min. — Sampled at 21, 23, 25, 28, 31, 35, 40, 50 min. — Sampled at 40, 50 min. Supernatant diluted 1/2000 and 0·5 ml. assayed with 5 min. adsorption on *Bact. coli* B.

Effect of phosphate on phage adsorption.

It has been shown by Cohen (1948) and by Kozloff and Putnam (1950) by isotope methods that the greater part of the phosphate in the desoxyribonucleic acid of the T series of phages comes from the medium and not from bacterial nucleic acid, and that this is the essential role played by phosphate in phage multiplication. It seemed worth while, however, to determine whether phosphate had any effect on the adsorption of phage to bacteria. In one experiment *Bact. coli* B was grown for 4 hours in S-medium, washed twice in synthetic medium free from phosphate and re-suspended in the same medium containing 0·1 M 4(5)-methylglyoxaline hydrochloride as buffer (pK 7·52). The culture

was divided into three portions and phosphate buffer (pH 7·5) added to varying concentrations, the total salt concentration being kept constant by adding an ionically equivalent solution of NaCl. Phage T2 in S-medium was added and the course of adsorption followed as usual (Fig. 5). It appeared that at low concentrations of phosphate adsorption of the phage was inhibited, the limiting

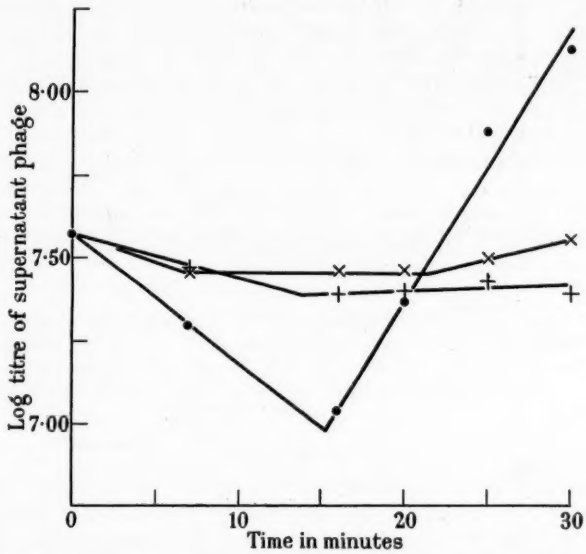

FIG. 5.—Effect of phosphate concentration on adsorption and multiplication of T2 phage. Organisms grown in S-medium, washed twice and resuspended in S-medium without phosphate. In each tube 16 ml. *Bact. coli* B suspension + 2 ml.M 4(5)-methyl-glyoxaline hydrochloride + 1 ml. diluted T2 phage.

●——●, + 2 ml. 0·1M phosphate, pH 7·5. Final phosphate concentration, 0·011M.

+——+, + 0·2 ml. 0·1M phosphate, pH 7·5 + 1·8 ml. 0·23M NaCl. Final phosphate concentration, 0·002M.

×——×, + 2·0 ml. 0·23M NaCl. Final phosphate concentration, 0·001M.

Bact. coli B, $6·7 \times 10^7$ viable organisms per ml. Initial titre of T2 phage, $3·7 \times 10^7$ lytic particles per ml.

concentration of phosphate in several experiments being about 0·004M. However, to ensure that the inhibition was not in any way due to the presence of 4(5)-methylglyoxaline hydrochloride, the experiment was repeated in its absence (Fig. 6). Adsorption and release of phage occur normally down to 0·001M phosphate; there is some evidence of inhibition in the complete absence of phosphate, but the final pH of this mixture was below 5, so that the result cannot be considered significant. It is apparent that any inhibitory effect in previous experiments was due to the glyoxaline, and that this effect could be reversed by phosphate. This result may be of some interest in view of the presence of a glyoxaline ring in histidine.

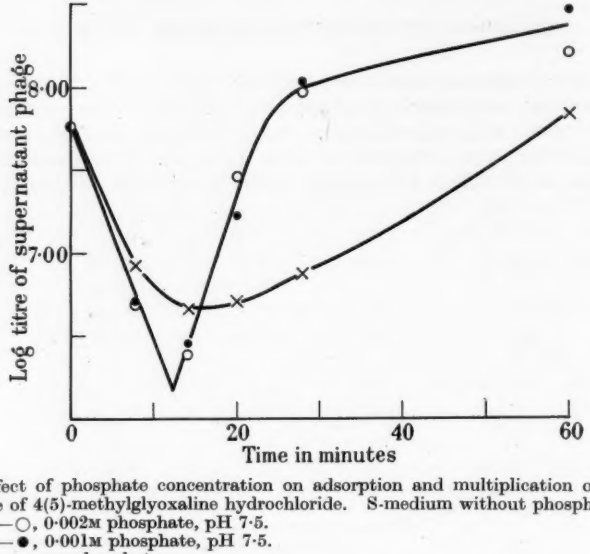

FIG. 6.—Effect of phosphate concentration on adsorption and multiplication of T2 phage in absence of 4(5)-methylglyoxaline hydrochloride. S-medium without phosphate.
○———○, 0·002M phosphate, pH 7·5.
●———●, 0·001M phosphate, pH 7·5.
×———×, no phosphate.
Bact. coli B, $6{\cdot}2 \times 10^7$ viable organisms per ml. Initial titre of T2 phage, $5{\cdot}8 \times 10^7$ lytic particles per ml.

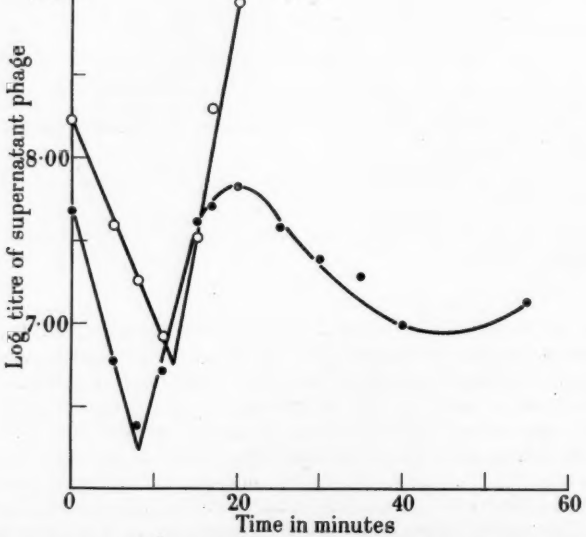

FIG. 7.—Adsorption and elution of T2 and T3 phages added simultaneously to *Bact. coli* B. GTP-medium. *Bact. coli* B, $7{\cdot}7 \times 10^7$ viable organisms per ml.
○———○, T2 phage. Initial titre, $1{\cdot}7 \times 10^8$ lytic particles per ml.
●———●, T3 phage. Initial titre, $4{\cdot}8 \times 10^7$ lytic particles per ml.

Adsorption of a mixture of phages.

When a mixture of two phages is added to a culture of organisms susceptible to both, it is well-known that, under certain conditions, multiplication of one is almost completely inhibited, while that of the other is only slightly reduced; moreover, it is not necessarily the one with the longer latent period which is inhibited. In the case of the T2 and T3 phages, if T2 is added at the same time as, or up to two minutes after, T3, it is the T2 phage which multiplies (Delbrück and Luria, 1942), although its latent period in " single-step " experiments is 21 min. as compared to 13 min. for T3. It therefore seemed of interest to determine whether the adsorption and release of the phages were affected in such a mixture. An experiment was set up in the usual way, except that a mixture of T2 and T3 phages was used, the T2 being present in excess to ensure that all bacteria had adsorbed phage. Alterations in titre of the supernatant phages T2 and T3 were followed separately by plating out on *Bact. coli* B mutants resistant to phages T3 and T2 respectively. Results are shown in Fig. 7, and it is apparent that adsorption of both phages occurs normally. The maximum titre of T2 is 16·4 times the initial titre and that of the T3 1·4 times. More than one lytic particle must have been adsorbed per bacterium, since the total of particles disappearing from the supernatant during the adsorption is more than 2×10^8 per ml., whilst the number of bacteria present is $7{\cdot}7 \times 10^7$ per ml. But more noteworthy is the fact that adsorption of the T3 phage is complete, and release of the phage has begun 3·5 min. before adsorption of T2 phage is complete; nevertheless it is the T2 which multiplies, not the T3.

DISCUSSION.

The results described in this paper show, not merely that adsorption of T2 phage on to *Bact. coli* B is logarithmic, but that so also is its release during the early stages of multiplication, at least under the conditions of these experiments. It is not wise to draw conclusions from a complex system in which multiplication of the virus and its release from the bacterial cell are not necessarily closely linked, but a logarithmic rate of increase would be expected if the phage is a self-duplicating unit. Cohen (1947) found that the rates of increase of phage protein and desoxyribonucleic acid were linear and not logarithmic, and it is presumably this rate which would ultimately control that of the virus release into the medium. On the other hand, recent isotope work (Evans, 1950) indicates that a portion of the original phage inoculum (perhaps part of its nucleic acid) is incorporated in each of the phage particles produced, from which one might conclude that the process is one of self-duplication. Further information is obviously required before a clear picture of the process can be obtained.

In the interpretation of " single-step " experiments the assumption is usually made that during the latent period, when the phage titre is constant, the phage after penetration of the bacterial cell-wall multiplies inside the bacterium so that, although there is an increase in the number of phage particles, the titre appears the same because all the phage particles inside one bacterium will produce only a single plaque on plating out. At the end of the latent period the infected bacteria begin to burst and release their phage, producing the rise in titre. The experiments reported in the present paper, however, show that T2 phage may be released after only 12–14 minutes, although the latent period of " single-step "

experiments is 19–21 minutes. Probably the simplest of many hypotheses to explain this discrepancy is that the physical state of the infected bacteria has been so affected by phage multiplication that mere dilution into phosphate-saline is sufficient to release the phage from the bacterial residue. It has been shown by Fowler and Cohen (1948) that addition of certain amino-acids to a synthetic medium will influence cellular lysis by T2 phage without effect on formation of the phage, and by Price (1949) that lysis of *Staphylococcus muscae* by a phage may or may not accompany virus liberation, depending on conditions in the medium. It also appears that the fundamental changes inside *Bact. coli* B during formation of T2 phage are complete about 12 minutes after infection; after this time 5-methyl-tryptophane will no longer completely inhibit virus liberation (Cohen and Fowler, 1947), whilst changes in resistance of the virus to ultra-violet light and to X-rays show that multiplication is completed about the 13th minute (Luria and Latarjet, 1947; Latarjet, 1948). The reason for this delay in release of phage from the bacteria is not clear. Latarjet has suggested that the remainder of the latent period in "single-step" experiments is spent in rearrangement of the phage protein and that bacteria burst when this process is complete, but such an hypothesis only accords with the present results if it is assumed that protein synthesis of phage can continue after it has been freed from the bacterial residue.

The need for caution in interpreting the relation between phage adsorption and release and its multiplication is stressed by the results of the experiment with a mixture of two phages. The early part of the curve for each phage is identical with that obtained when the phage is tested alone, although both T2 and T3 phages must be adsorbed on the same host cell in some cases; presumably the two require different receptors on the surface of the bacteria. By what means the multiplication of the more rapidly adsorbed T3 is suppressed by the T2 phage is not clear, but the method employed in these experiments provides a means whereby this problem can be investigated.

SUMMARY.

A method is described for following the course of adsorption and multiplication of phages by assay at intervals of the supernatant phage after centrifugation of the bacteria with their adsorbed phage. Application of the method to the T2 coliphage shows that logarithmic adsorption occurs up to 12–14 minutes, after which there is logarithmic release of the new phage formed. When a mixture of T2 and T3 phages is employed the adsorption curves of the two viruses are identical with those of the phages separately, but only the T2 phage multiplies.

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STUDIES ON AGGLUTINATION OF RED CELLS BY *CLOSTRIDIA*.

I. *CL. SEPTIQUE*.

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THE ability of bacterial suspensions to agglutinate red cells was first observed by Kraus and Ludwig in 1902. These authors reported that certain staphylococci and vibrios were able to agglutinate rabbit red blood cells. They also observed that this haemagglutination could be inhibited by the action of specific antiserum. Similar observations with *Bact. coli* were reported by Guyot (1908) and with *C. diphtheriae* and certain species of *Salmonella* by Fukuhara (1909). These early observations received little attention until 1941, when Hirst described agglutination of fowl red cells by influenza virus. Since then agglutination of red cells by viruses has been extensively studied. Recently Keogh, North and Warburton (1947*a*) described agglutination of red blood cells by saline extracts of *H. pertussis*, *H. parapertussis* and *H. bronchisepticus*. Similar observations were made by Middlebrook and Dubos (1948) with extracts of tubercle bacilli, and by Kauffmann (1948) with suspensions of *Bact. coli*. Our studies are concerned with the capacity of toxins and suspensions of pathogenic *Clostridia* to agglutinate erythrocytes of various species of animals. Experiments were also carried out to ascertain whether agglutination could be inhibited by a specific antiserum.

MATERIALS AND METHODS.

Preparation of erythrocytes.—In most instances ox erythrocytes have been used, but red cells of the horse, sheep, goat, rabbit, guinea-pig and fowl have also been investigated.

Because of the haemolytic action of some *Clostridia* the red cell suspension has to be treated with an agent which will prevent haemolysis by the toxin or bacterial suspension. Preliminary studies were made with erythrocytes treated with formalin. The effect of different concentrations of formalin on red cells will be reported in another paper. The following method was found satisfactory for the purpose of this investigation :

Erythrocytes were added in 2 per cent volume to modified Alsever's solution containing—

Glucose	4·66 g.
Sodium chloride	1·05 g.
Sodium citrate	2·00 g.
Distilled water	200·00 ml.

Formalin was then added to make a final concentration of 2 per cent. This suspension was kept in the refrigerator for five days and shaken every day.

* Seconded from the Sudan Veterinary Services, Khartoum.

After the fifth day the supernatant fluid was decanted and the red cells were re-suspended in Alsever's solution containing 0·3 per cent formalin. At the beginning of each experiment the preserved erythrocytes were washed and then suspended in 1 per cent saline.

Organisms tested.—Three strains of *Cl. septique* were examined. They were usually grown either in Robertson's cooked meat medium or on 5 per cent horse blood agar plates.

Toxins.—In some experiments the supernatant fluids from centrifuged cooked meat broth cultures were used, and in others solutions of precipitated toxin of *Cl. septique*.

Antisera.—Antisera against *Cl. septique* were obtained from rabbits, which had been hyperimmunised with the whole culture of *Cl. septique* grown in Robertson's cooked meat medium. Standard antitoxic sera were obtained from the Wellcome Laboratories.

EXPERIMENTAL.

Effect of Cl. septique *Culture on Red Cell Suspensions.*

After preliminary experiments to ascertain whether it would be possible to sensitize red cells with *Cl. septique* culture in a fluid medium and then render them agglutinable by adding specific antiserum, it was noticed that formalised red cells could be agglutinated by the culture of *Cl. septique* alone. Further trials showed that the following method of performing the test was satisfactory. Serial twofold dilutions (volume 0·5 ml.) of a 48-hour-old culture in cooked meat medium were made in saline and were mixed with an equal volume of 1 per cent formalised ox red cell suspension. The mixtures were incubated for one hour at 37° and left at room temperature overnight. The test was read as recommended by Salk (1944) for haemagglutination by influenza virus.

In control tubes containing red cells and saline the sedimented cells formed a compact button at the bottom of the tube. In the mixture of red cells and bacterial culture the cells were loosely aggregated, giving the impression that the clumps of cells had been dispersed rather than aggregated and had then adhered to the bottom of the tube. The last dilution showing definite haemagglutination was taken as the end-point in the titration.

Since it was observed that the capacity of the bacterial culture to agglutinate red cells was enhanced in the presence of normal serum, 0·1 ml. of normal rabbit serum was added to each tube containing the mixture of culture and red cell suspension. It must be pointed out, of course, that normal rabbit serum has itself no agglutinating effect on the ox red cells. The effect of normal sera from certain species on different red cell suspensions will be discussed later.

TABLE I.—*The Effect of Fluid Culture of* Cl. septique *on Erythrocytes of Several Species of Animals.*

Origin of red cells.	Dilutions of *Cl. septique* culture.					
	1:4.	1:8.	1:16.	1:32.	1:64.	1:128.
Ox	+	+	+	+	+	−
Sheep	+	+	+	+	+	−
Horse	+	+	+	−	−	−
Rabbit	+	+	+	+	−	−
Guinea-pig	+	+	+	+	−	−
Fowl	+	+	+	+	+	−

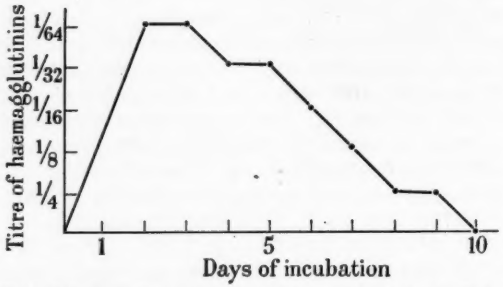
+ = Complete agglutination. − = No agglutination.

To determine whether red cells of animals other than the ox can be agglutinated by *Cl. septique* culture those of the following were tested : sheep, horse, rabbit, guinea-pig and fowl. The best results were obtained with ox, sheep and fowl red cells.

Nature of the Agglutinating Factor.

Media.—Attempts were made to find the medium which was most favourable for the production of haemagglutinins. Cultures in Robertson's medium (with and without glucose), Brewer's medium containing liver and sodium thioglycollate and blood agar were tested daily for 10 days. There was little difference in the amount of haemagglutinin produced, but cooked meat medium and blood agar gave slightly higher titres than the other media. The presence of glucose appeared to reduce the amount of haemagglutinin produced, probably owing to acid production.

Time.—The period of incubation is important in the production of haemagglutinin. It was found that a 24- to 48-hour culture in cooked meat medium or liver broth produced the maximum haemagglutinin, which thereafter fell until no significant agglutination could be detected after ten days' incubation at 37° (see graph).



Graph showing effect of time on the production of haemagglutinins in Robertson's medium.

Effects of Physical and Chemical Agents.

The haemagglutinin is thermolabile. Fluid cultures or bacterial suspensions heated at 60° for half an hour were completely inactivated. On the other hand, whole culture or bacterial suspension treated with either 0·5 per cent formalin, 0·5 per cent phenol or absolute alcohol for 24 hours were still fully active.

The pH of the fluid in which cultures were suspended was also of importance. Cultures on blood agar plates were suspended in saline at pH 4·5, 7·0 and 9·0, and incubated for 24 hours. After this time the cells were washed and tested for the presence of the agglutinating factor. It was found that at pH 4·5 the agglutinating factor was lost, whereas no appreciable drop in titre occurred at pH 7·0 or 9·0.

Supernatant fluid and bacterial cells.—Experiments were first made with whole culture in Robertson's medium, which contained both soluble substances and bacterial cells. To determine whether the factor responsible was in the bacterial cell or in solution, culture was centrifuged and both the supernatant and the sedimented bacterial cells were tested for haemagglutinins. The supernatant

was inert or produced only a slight reaction, whereas the sedimented bacterial cells induced strong haemagglutination. It is evident, therefore, that most of the activity is associated with the bacterial cell. It remained to be determined whether agglutination was produced by the bacterial bodies or by a soluble fraction adherent to them which might be removed by washing. The sedimented bacteria were washed five times and each supernatant was tested. These had no effect on red cell suspension, whereas the bacterial cells after five washings showed only a slight loss of haemagglutinin.

Toxins.—It thus appears that the bacterial cells were mainly responsible for the haemagglutination reaction. In order to confirm the inactivity of toxin towards red cells the precipitated toxin was examined. The minimal lethal dose of the toxin as determined by intravenous inoculation was 0·0005 g. for a mouse of 20 g. Dilutions were then set up containing 1, 10 and 100 lethal doses of toxin. No agglutination occurred when red cell suspension was added. It was also found that the precipitated toxin did not render red cells agglutinable by the addition of antiserum, as described by Middlebrook and Dubos (1948) with extracts of tubercle bacilli.

Agglutinin-Inhibition Reaction.

The ability of anaerobic organisms to agglutinate red cell suspension is of little practical value unless the reaction can be inhibited specifically by the corresponding antisera. Difficulties arise in performing haemagglutination inhibition tests owing to the presence of normal agglutinins for red cells in the sera of different species. It was therefore necessary to make a preliminary study with normal sera and red cells in order to find a system in which low dilutions of normal serum would not agglutinate the red cell suspension.

Haemagglutination by normal sera.—Serial dilutions of normal sera of various species of animal ranging from 1:4 to 1:1024 were mixed with homologous and heterologous red cell suspensions in saline.

TABLE II.—*The Effect of Normal Sera on Formalized Erythrocytes.*

Erythrocytes.	Normal serum.				
	Ox.	Sheep.	Horse.	Rabbit.	Guinea-pig.
Ox	1/4*	—	—	—	—
Sheep	1/8	1/4	—	—	1/32
Horse	1/32	1/16	1/8	1/16	1/4
Rabbit	1/512	1/32	1/8	—	1/64
Guinea-pig	1/8	1/16	1/8	1/512	1/8
Fowl	1/16	1/16	1/16	1/16	1/8

* Fractions represent the last dilution of serum showing definite agglutination.
— = No agglutination.

The results show that ox and sheep cells were the least sensitive to the normal agglutinins in the sera studied. As the antisera against the anaerobic organisms were obtained either from horses or from rabbits, it is clear that a system using ox red cells is the most suitable since it obviates any possible confusion through non-specific agglutination.

Action of specific antisera.—The absence of any significant agglutination by non-specific factors in serum permitted experiments to be undertaken on agglutinin inhibition reaction by specific antibacterial and antitoxic sera.

In the haemagglutinin inhibition test with influenza virus it is immaterial whether the immune serum is diluted directly in the red cell suspension and virus added to this mixture, or whether the serum is diluted in saline and then virus and cells subsequently added. In the present studies it has been found that a higher titre of inhibition was obtained when 1 per cent erythrocytes and serial dilutions of antiserum were incubated for half an hour and then the antigen added. The following method has therefore been used for the inhibition test: To serial twofold dilutions of antiserum in saline in a volume of 0·5 ml. are added 0·5 ml. of a 1 per cent suspension of formalized red cells. The mixtures are incubated at 37° for half an hour. Each tube is then completed with 0·5 ml. of bacterial culture containing four haemagglutinating doses. The tubes are incubated at 37° for 1 hour and then left overnight at room temperature. A duplicate set is put up with normal serum in place of antibacterial or antitoxic serum. Controls of red cells and culture and red cells and saline are also included. As ox red cells sediment at a slower rate than fowl cells, complete sedimentation takes from 3 to 4 hours at room temperature. The pattern of the sedimented cells is unchanged after 24 hours.

TABLE III.—*Haemagglutination Inhibition Test with* Cl. septique *Culture.*

Type of antiserum.	Dilutions of tested sera. 1/6.	1/12.	1/24.	1/48.	1/96.	1/192.	1/384.	1/768.	1/1536.
Cl. septique (antitoxic)	−	−	−	−	−	−	−	++	+++
Cl. septique (antibact.)	−	−	−	−	−	++	++	+++	+++
Cl. chauvoei	++	++	++	++	++	++	++	+++	+++
Cl. welchii D.	++	++	++	+++	+++	+++	+++	+++	+++
Cl. oedematiens B.	+++	+++	+++	+++	+++	+++	+++	+++	+++
Normal rabbit serum	+++	+++	+++	+++	+++	+++	+++	+++	+++

It is of interest that although pure toxins have no effect on ox erythrocytes, antitoxic sera have the same inhibitory effect on haemagglutination as antibacterial sera. It is possible that a factor responsible for the inhibition of agglutination is common to both antitoxic and antibacterial sera, but its nature and its relation to the antitoxic and antibacterial immune bodies has yet to be determined.

DISCUSSION.

The experiments recorded in the paper show that formalised red cells of different species can be agglutinated by cultures of *Cl. septique*.

The reaction is more akin to the agglutination of red cells by viruses, and differs from the haemagglutination reaction described by Keogh, North and Warburton (1947) or Middlebrook and Dubos (1948). In the method proposed by these authors, sheep or ox erythrocytes were first sensitized by the adsorption of components of bacteria on erythrocytes, which were thus rendered agglutinable by specific antisera. It has been suggested by Keogh *et al.* (1947*b*) and by Middlebrook and Dubos (1948) that polysaccharides are the components involved in direct sensitization of erythrocytes. On the other hand Griffiths (1948) showed that erythrocytes can be agglutinated directly by *Shigella alkalescens* culture containing haemagglutinins. He was also able to separate the haem-

agglutinating factor from the bacterial cell by high-speed centrifugation after successive freezing and thawing of bacterial suspensions. Our experiments with *Cl. septique* have shown that the agglutinating factor is associated with bacterial cells, but not with toxins, since the latter were unable to agglutinate red cells or to sensitize them so that they were agglutinated by antitoxic sera. It has been found that the aggultinating factor of *Cl. septique* is thermolabile, but it is not destroyed by chemicals such as 0·5 per cent formalin, 0·5 per cent phenol or 95 per cent ethyl alcohol. Similar observations were recorded by Griffiths (1948).

The agglutinating factor can be inhibited by specific antibacterial or antitoxic sera. A haemagglutination inhibition test based on the technique described by McClelland and Hare (1941) for influenza virus has been elaborated. Since the toxin of *Cl. septique* was incapable of causing agglutination of red cells, it was anticipated that antitoxic sera as opposed to antibacterial sera would not be capable of neutralizing the haemagglutinin. It is therefore surprising that antitoxic sera had the same inhibitory effect as antibacterial sera. At the moment we are unable to offer any satisfactory explanation of this curious phenomenon, and can do no more than suggest that both sera contain antiagglutinable factors.

Similar studies are being made with *Cl. welchii* and other species of *Clostridium*. If their results are the same as those with *Cl. septique*, the haemagglutination inhibition test may have some practical application in the identification of organisms in this genus.

SUMMARY.

Cultures of *Cl. septique* agglutinate erythrocytes of various species of animal in a manner similar to that of influenza virus. Haemagglutination can be neutralized by specific antisera.

The agglutinating factor is associated with the bacterial cell, but not with the toxins; it is thermolabile, and is not destroyed by 0·5 per cent formalin, 0·5 per cent phenol or absolute alcohol.

We are indebted to Dr. C. L. Oakley for supplying us with dry toxins of *Cl. septique* and for his interest in the work, and to Professor R. E. Glover for his valuable suggestions, and encouragement during these investigations.

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THE HISTOLOGY OF THE TUBERCULIN REACTION AND ITS MODIFICATION BY CORTISONE.

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THE tuberculin reaction is usually taken as the typical example of an " allergic " tissue reaction, one, that is, characterised by cellular exudation, in contrast with the histamine-anaphylactic type, characterised by a vascular reaction without marked or lasting infiltration by cells.

The remarkable activity of A.C.T.H. and of cortisone in suppressing allergic manifestations has been shown clearly by their effect on the tuberculin reaction in guinea-pigs (Long and Miles, 1950), rabbits (Harris and Harris, 1950) and man (Long and Favour, 1950). Cortisone has, however, an equally striking effect on other, non-allergic tissue reactions ; it delays, for instance, the healing of simple wounds (Ragan, Howes, Plotz, Meyer and Blunt, 1949) and fractures (Blunt, Plotz, Lattes, Howes, Meyer and Ragan, 1950). The question arises whether suppression of an allergic reaction is merely an example of this general non-specific inhibition of tissue responses, or whether there is in addition a specific anti-allergic component.

An attempt has been made therefore to analyse the difference in the histology of these two kinds of reaction, namely, the non-specific response to injury and the specific response to tuberculin in sensitised animals and to interpret their modification by cortisone in the light of this analysis. Then, by comparing the characteristic effects of cortisone on two reactions of quite different pathogenesis, it was hoped to isolate the specific anti-allergic activity of cortisone, if any, and also to throw light upon the relationship between the two types of tissue reaction.

METHODS AND MATERIALS.

Since well-marked suppression of the tuberculin reaction occurs in sensitised rabbits given cortisone (Harris and Harris, 1950), we selected this animal for detailed investigation, in preference to the guinea-pig where the suppression is less striking.

Method of sensitisation.

After some preliminary experiments, eight young adult sandy-lop rabbits (M.R.C. strain) were sensitised by intradermal injection between the scapulae of 0·3 mg. dried heat-killed tubercle bacilli (human type), suspended in 0·03 ml. liquid paraffin.

Tuberculin testing.

From experiments on these and other groups of animals, the most suitable dose was found to be 80 International Units (0·1 ml. of a 1/125 dilution in saline) of Old Tuberculin (kindly supplied by the National Bureau of Standards). This dose gave a histological reaction of sufficient intensity, but without necrosis, in sensitised animals, and only a negligible tissue reaction in normals.

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Non-specific inflammatory reaction.

After trials with a number of irritants, an approximately 0·2 per cent suspension of crude guinea-pig brain phospholipid (chloroform-soluble acetone-insoluble fraction) was chosen. This gave a reaction roughly comparable in size with that to 80 I.U. tuberculin in the sensitised animal at 22 hours, also without necrosis.

Dosage of cortisone.

In the preliminary experiments various dosage schedules of cortisone were used, always, however, giving at least one dose of 30 mg. Extra doses of 10–15 mg., 46 to 18 hours before biopsy, made no apparent difference to the results, and the second dose given in the experiment described in detail below was probably unnecessary.

Treatment and biopsy of animals.

After preliminary tests, cortisone being used on some animals of the group and biopsies taken as described below, two pairs of animals were selected, one pair being moderately sensitive (R.55, R.58) and the other pair strongly so (R.54, R.57), as judged by the sizes of the tuberculin reactions. Three weeks after the last testing (nine weeks from the original sensitising injection) one animal from each pair (R.57, R.58), i.e., a strongly and a moderately sensitised animal, was given 30 mg. (10–12 mg./kg.) of cortisone acetate (Merck) subcutaneously. Four hours later all four animals and one normal non-sensitised animal were given two intradermal injections, one on each side, of 80 I.U. Old Tuberculin in 0·1 ml. saline, in the previously shaven lumbar region about 4 cm. from the mid line, and at the same time two injections of 0·1 ml. of the phospholipid suspension (0·2 per cent) about 5 cm. caudally. The centre of each injection-bleb was marked with a spot of dye. After a further four hours one tuberculin lesion and one phospholipid lesion were removed from each animal under local anaesthesia. The biopsy specimen, measuring about 2·5 × 0·5 cm., comprised the whole of the cutis vera, and included the centre of the lesion as judged by the dye spot, and a small piece of normal skin at either end.

A further subcutaneous injection of cortisone (10 mg.) was given to the two animals previously treated (R.57, R.58) when the biopsies were finished, and the next day, twenty-two hours after the injections of tuberculin and phospholipid, the remaining pairs of lesions in all animals were removed.

Twelve days later all the sensitised animals were tuberculin-tested and the comparable sensitivities of the pairs of animals used were confirmed.

Histological methods.

The biopsy specimens were placed overnight in mercury-acetic fixative (sat. $HgCl_2$ with 3 per cent acetic acid), bisected longitudinally, dehydrated in alcohol, cleared in methyl salicylate, and after removal of the latter by benzene, embedded in paraffin. Sections were cut at 5 μ, in most cases at two or more levels from the inner face of the separate halves of each specimen, and stained routinely with haematoxylin and eosin, and by other methods as required—Unna-Pappenheim, van Gieson, and the reticulin method of Caldwell and Rannie (1947).

Cell counts.

Differential cell counts were made on sections to confirm subjective impressions of the relative proportions of the infiltrating cells, and the changes that occur in these as the lesion develops.

Tuberculin reaction.—(A) Differential counts were made on ten separate high-power fields centred round the site of maximum reaction, each field being chosen midway between consecutive hair-follicles and about one-half the diameter of the high-power field below the epidermis. This was judged the most suitable zone in the 4-hour lesion to define the changes in cell population due to cortisone, since at this time, although the polymorphonuclear leucocytes (polymorphs) have a fairly uniform distribution, the mesenchymal reaction and mononuclear infiltration is, apart from the perivascular foci, largely confined to this superficial strip; possible reasons for this are discussed later. The polymorphs were counted against all other cells of mesenchymal origin (fibroblasts, free and fixed histiocytes, and endothelial cells). Classification of the latter group of cell-types is difficult, and unnecessary if, as here, it is only desired to determine changes in the relative proportions of granular and non-granular cells.

A similar count was made on the lesion produced by the injection of tuberculin into a non-sensitive animal. This supplies a more suitable baseline than counts from normal skin, since allowance is made for the separation of the local cell population by the injection fluid, and for the slight non-specific reaction. If these baseline figures are subtracted from the counts on the experimental animals, a more precise idea is obtained of the changes in the reaction due to sensitisation, and their modification by cortisone.

Counts were also made by the same method on the 22-hour lesions. Since the characteristic perivascular foci of mononuclear cells are minimal in the zone counted, the method now underestimates the number of these cells. The counts do however confirm the visual impression of the effect of cortisone on the diffuse mesenchymal-cell reaction, and, by comparison with the previous counts, the changes in polymorphonuclear infiltration of the superficial dermis both in control and cortisone-treated animals are made evident.

(B) The perivascular infiltrations in the 4- and 22-hour lesions were also analysed, by differential counts of the cells contained in one high-power field centred on a suitable small blood-vessel. Twenty such foci were counted from each lesion, ten in the superficial dermis and ten in the deep areolar tissue. Since it was not possible to select twenty comparable vessels in all four animals, these figures can only be used to demonstrate alterations in the proportion of granular and non-granular cells following treatment by cortisone, and not changes in the absolute numbers of each class of cell.

Brain-phospholipid lesions.—The irregular distribution of the cells made it impossible to select truly comparable areas, and so to compare the intensity of polymorph reaction in different animals by cell-counts, but differential counts of the perivascular infiltrations were made by the method described under (B) above.

HISTOLOGICAL FINDINGS.

In the following report the description " mononuclear cell " is frequently used as a comprehensive term for all free non-granular cells, irrespective of their possible origin from local histiocytes or from invading lymphocytes and monocytes of the blood. The term " histiocyte " is reserved for cells which we feel justified in assuming to be normally resident in the tissue studied; they correspond to the resting wandering cells of Maximow. While those cells when stimulated may of course become mobilised as large amoeboid wandering cells or " macrophages," a large proportion of exudate mononuclear cells are monocytes and lymphocytes which have emigrated from the blood-stream. It is therefore reasonable to assume that in the lesions studied, the smaller rounded mononuclear cells (identical in appearance with the monocytes of the blood) which are most numerous in the early stages, especially in the region of thin-walled blood-vessels, are actually migrated haemic cells. Such cells are also considered to develop into the larger amoeboid type of wandering cell, becoming indistinguishable from those originating from local tissue cells. In the older lesions examined these larger forms predominate, and the origin of any particular one cannot be determined from its appearance alone. The name " macrophage," which is commonly applied to these free cells, is not used, since it implies a functional activity, potential no doubt in the free mononuclear cells present in the tuberculin reaction, but not, we believe, the primary function of the majority. The large pale cells most frequent near small blood-vessels, which become increasingly prominent as the lesion matures, may be identified with the " undifferentiated mesenchyme cell " of Maximow, and as such are assumed to contribute to the mononuclear cell infiltration.

We are aware that the reaction in the rabbit differs in detail from the lesions produced in other species owing to variations in the intimate structure of the skin, but the fundamental process is the same in all.

Local Reaction Following Intradermal Injection of Tuberculin in Sensitised Rabbits.

The tuberculin reaction shows all the separate cellular, vascular and humoral components of a non-allergic acute inflammatory response, but it differs in its characteristic pattern and in the quantitative and time relations of the individual components.

Macroscopically, with the dose of tuberculin used, swelling and hyperaemia are insignificant at 4 hours, but by 22 hours the lesion is demarcated by palpable swelling and uniform erythema. It would appear from the sections that this swelling is largely due to increase in cellularity, since at no time is there any marked capillary exudation and the swelling due to the injection fluid is by this time largely dispersed.

Microscopically the reaction involves the whole thickness of the dermis, and when shreds of the underlying fascia have been included is as great here as anywhere in the true dermis. The pattern of the response consists essentially of two components, a generalised increase in cellularity without any definite centre of maximum intensity, and a multifocal reaction round thin-walled blood vessels; its intensity is characteristic for each structural layer of the dermis.

4-*hour lesion.*—Many of the small arteries, arterioles, capillaries and venules, and lymphatics are dilated. The dilatation of the lymphatics and venules is probably in part a reaction to the increased fluid drainage incidental to the injection, since it, like the oedema in the compact layer of the dermis, is of comparable intensity in a control lesion in a non-sensitised animal. A specific vascular reaction is, however, undoubtedly present, as shown by the more conspicuous capillary dilatation and by the small but definite serous exudate not present in the control lesion.

The perivascular cellular infiltration is only as yet of slight to moderate intensity, the hyperplasia of the endothelial and perithelial cells contributing to the prominence of the vessels. The infiltrating cells consist of mononuclear cells, some small, with well-defined outline resembling the monocytes of the blood but with all transitions to larger cells of more histiocytic appearance, polymorphs, and a few lymphocytes (Fig. 1*a*). Although at first sight polymorphs may appear to predominate in the perivascular infiltrations, this is most often a false impression from the large number of these cells within the dilated vessels; outside the vessel wall they may be hardly more numerous than in the surrounding diffuse infiltration (see Table I, B, 1). Small groups of the larger histiocytic cells (some probably of local origin) frequently lie in direct contact with the outer surface of a vessel wall, forming an incomplete ring or a small focal collection at one point on its circumference.

The diffuse cellular reaction is in part a true infiltration of polymorphs and free mononuclear cells, and in part an increased prominence and hyperplasia of the native mesenchymal cell population, most noticeably of the histiocytes and adventitial cells of Marchand. Polymorphs are the commonest type of infiltrating cell, and their density is characteristic for any one morphological layer of the dermis. They are most numerous in the loose areolar tissue, and only in the superficial dermis are they outnumbered by the new mononuclear cell population (see Table I, A, 1). Their distribution in any one structural layer is uniform (apart from a gradual centripetal increase in density), without any significant

focal concentrations, even to any marked extent round the blood-vessels. Occasional rather dense accumulations in the compact dermis are almost certainly due to injection trauma, since in suitable sections from sensitised and control animals they can be seen to lie along the line of the needle track. The degenerate appearance of many of the polymorphs and their diffuse distribution suggest that the polymorph reaction is already near its peak at 4 hours. The mononuclear cell component of the diffuse cellular reaction is also characteristic for the different structural layers of the dermis, but in contrast with the polymorphonuclear, is most pronounced in the superficial dermis, where the vascularity and the relative abundance of mesenchymal cells provide the best opportunity for both cell infiltration and hyperplasia. Elsewhere, the effect of hyperplasia of local tissue cells is insignificant owing to their natural scarcity, and small free mononuclear cells, presumed to be of haemic origin, are very largely confined to the neighbourhood of blood vessels. The result of this is, in the rabbit, a characteristic band of maximum cellularity in the superficial dermis, with scattered perivascular foci in all the dermal layers.

22-hour lesion.—The picture is now one of histiocytic hyperplasia with the formation of characteristic perivascular accumulations of cells (Fig. 2*a*), so that mononuclear cells are more numerous than the remaining degenerate polymorphs. Oedema is largely limited to the superficial dermis, and while the lymphatics are widely dilated, capillaries are less noticeable. This is to some extent due to the large number of leucocytes, many of them mononuclear cells, in the lumina of the vessels, so that a capillary is difficult to distinguish from the surrounding accumulation of mononuclear cells. In extreme cases the lumen is so plugged with cells that it is unlikely that there is any effective circulation.

The perivascular collections of cells are dense and sharply outlined in the compact dermis, but more diffuse in looser tissue where they merge into the diffuse mononuclear cellular reaction. The morphology of the cells is not uniform (Fig. 3*a*); the majority can be classed as histiocytes, but in addition to a few lymphocytes and monocytes there are transitions to cells closely resembling the "activated" reticulum-cell described by Marshall and White (1950) in the lymph-nodes of animals subjected to repeated antigenic stimulation. Cells capable of forming reticulin fibres undoubtedly are present, since silver stains show that a fine reticulin net has already at 22 hours been laid down between the cells (Fig. 4*a* and *b*). While the origin of the various components of these perivascular aggregates cannot be decided from histological material only, their position and the picture in the earlier lesion suggests that both infiltrating and indigenous cells contribute to their formation, and the presence of mitotic figures in the 22-hour lesions is evidence that local proliferation is an additional factor in their later development. There are also occasional loose somewhat whorled collections of cells in the superficial dermis, containing lymphocytes and cells having the appearance of immature plasma cells (Fig. 2*a*). These may well develop from the small collections of lymphoid cells associated with conspicuous lymphatics which are to be found scattered beneath the epidermis in normal skin. The diffuse infiltration is now considerable, being most pronounced in the superficial dermis and in the deep loose areolar tissue. The same varieties of cells are present as in the perivascular foci, though their relative proportions differ. Blood monocytes are uncommon and almost confined to the vicinity of blood vessels, the majority of the mononuclear cells being larger and resembling the

wandering cells of histiocytic origin. However, in view of their abundance and the rarity of mitotic figures, we consider that a large proportion of them are hypertrophied blood monocytes, and that only where indigenous histiocytes and undifferentiated mesenchyme cells are naturally present in significant numbers, that is, in the superficial dermis and around blood vessels, does their proliferation add significantly to the mononuclear reaction. In addition to the wandering cells, there are, in the superficial dermis and to a minor degree elsewhere, all transitions to fixed histiocytes and reticulum cell types, especially around the perivascular cell accumulations.

The proliferation of the epidermis over the lesion is considerable and reaches 4–6 layers in the most severe reaction. No visible necrosis is present in either the epidermis or dermis.

From these observations of the local tuberculin reaction at 4 and 22 hours it is suggested that the essential features in the development of the mature lesion are an early and relatively transient infiltration by polymorphs and monocytes from the blood, followed by a proliferation and differentiation of the local histiocytes and undifferentiated mesenchyme cells and of the infiltrating blood monocytes.

Effect of Cortisone on the Reaction to Tuberculin.

Macroscopically, at 4 hours the effect of cortisone is not yet apparent, since even in the normal reaction swelling and hyperaemia are inconsiderable. In contrast, at 22 hours palpable swelling is either absent or very slight, and erythema if present is irregular and punctate rather than uniform.

In the microscopic sections there appears at first sight to be a comparable proportionate reduction in all the separate components of the tuberculin reaction, with the exception perhaps of the lymphatic dilatation and interstitial oedema. These are admittedly difficult to assess histologically, but it would seem that cortisone has little effect on their development at 4 hours, when they are probably to a large extent the result of the fluid injection. At 22 hours, however, while lymphatic dilatation persists in both control and cortisone-treated animals, the oedema in the superficial dermis, which is probably at this stage related to the vascular reaction, is considerably less in the treated animal.

Closer examination of the cellular reaction suggests that at 4 hours, although there are fewer polymorphs than in a control animal of comparable sensitivity, the difference is slight compared with the reduction of mononuclear cells (Fig. 1*b*). This is confirmed by the cell-counts, which show that in cortisone-treated animals both the total number and the proportion of mononuclear cells are decreased. The mononuclear reaction is almost confined to the immediate neighbourhood of blood vessels and to the superficial dermis ; the appearance of the cells involved suggests that this reaction is largely due to a hyperplasia of the local histiocytes and undifferentiated mesenchyme cells, small free mononuclear cells, of the type identified in the normal tuberculin reaction as infiltrating blood monocytes, being extremely rare.

At 22 hours, despite the marked suppression of the cellular and vascular reaction, the general pattern of the lesion as previously described in the control animals is still quite evident (Fig. 2*b*). The focal collections of mononuclear cells, although smaller, are still conspicuous and, with the exception of the small monocytic forms assumed to be of haemic origin, contain the same cell types as

do those of the control animal. Capillaries packed with white blood cells are not a feature, and mononuclear cells form only a small proportion of these.

The diffuse infiltration has the same distribution as in the untreated animal but differs in the greatly reduced number of large wandering cells, many of which in the normal lesion we consider to have developed from infiltrating blood monocytes, and in the larger proportion of degenerate polymorphs. The suppression of the mononuclear reaction is most marked in the acellular compact layer of the dermis, where normally infiltrating blood monocytes are almost entirely responsible for the mononuclear response ; while in situations where the proliferation of local tissue cells is an additional factor, the decrease in the intensity of the reaction is less, as in the perivascular foci and to a smaller extent in the superficial dermis. The absence of cells which can be identified as of haemic origin and the presence of a significant reaction in only those parts of the dermis where indigenous reactive cells are present in fair numbers suggests that the suppression of the tuberculin reaction by cortisone depends to a greater extent on the inhibition of cell infiltration than on the inhibition of cell hyperplasia.

The impression is gained that polymorphs are now more numerous than in the comparable control lesions (Fig. 3*b*), and that during the 4- to 22-hour interval not only has there been an increase in their proportion but their absolute number has tended to increase. This contrasts with their decrease in the control animal R54, and in other normal tuberculin lesions studied. The control animal R55 is however exceptional, in that the absolute polymorphonuclear count rises. However, the total infiltration in the 4-hour lesion in this animal is anomalously scanty when compared with that in the 22-hour lesion from the same animal ; this is probably the result of too deep an injection. If this anomalous lesion is omitted from consideration, it is evident that the polymorph count falls slightly in the untreated animal, but rises considerably in the animals treated with cortisone, between 4 and 22 hours. Two factors may combine to produce this effect. Firstly, cortisone may produce a delay in the mobilisation of polymorphs, as observed by Michael and Wharton (1951), so that they reach their maximum later than in the control lesions. Secondly, there is likely to be a failure of phagocytosis of effete polymorphs, as a natural consequence of the deficiency of mononuclear cells induced by cortisone. The unduly high proportion of polymorphs in 22-hour lesions, of low intensity due to treatment with cortisone, is in contrast with the balanced reduction of both cell types when a decrease in the intensity of the reaction is due to the low sensitivity of the animal tested, and is evidence that the action of cortisone in suppressing the tuberculin reaction is not simply equivalent to a reduction of the general sensitivity of the animal (Table I, B, 2 ; compare R57 and R58 with R55).

The modification of the tuberculin reaction by cortisone may therefore be

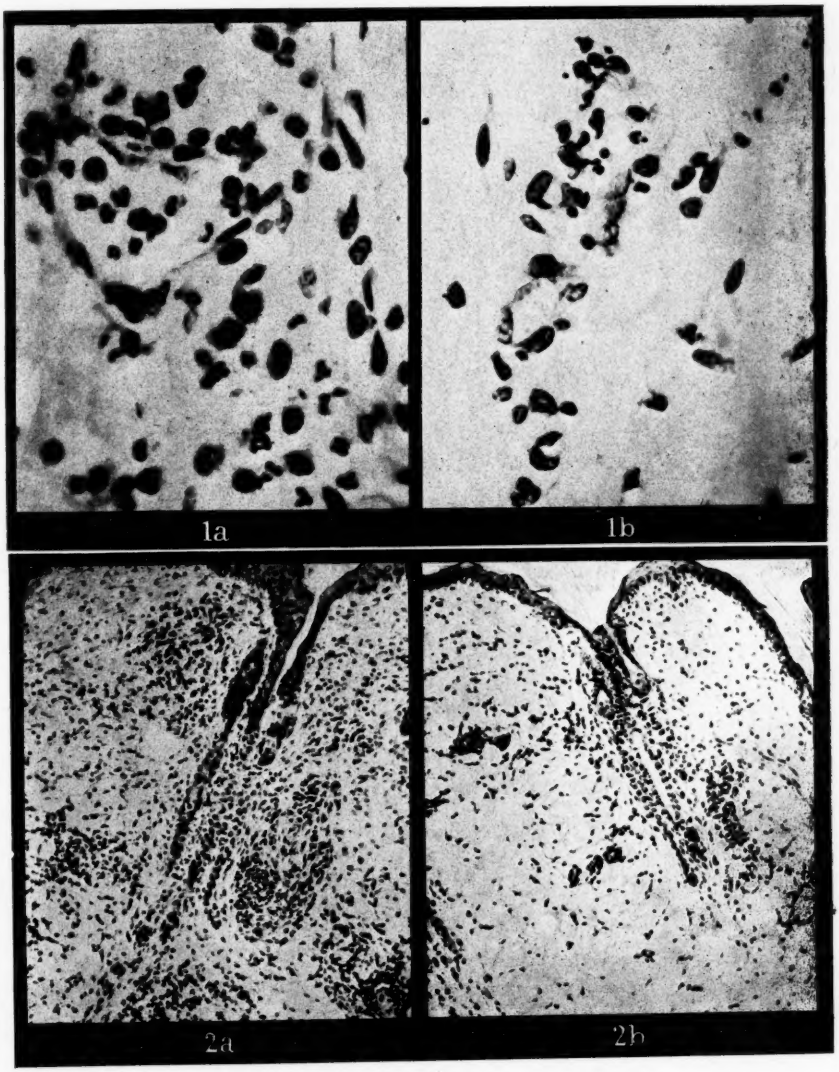
EXPLANATION OF PLATES.

FIG. 1.—Four hour tuberculin reaction : perivascular infiltration (*a*) in untreated rabbit, (*b*) in rabbit given cortisone. H. and E. × 544.

FIG. 2.—Twenty-two hour tuberculin reaction : superficial dermis (*a*) in untreated rabbit ; a collection of pyronin-positive cells is shown between the epidermis and the right hair follicle, (*b*) in rabbit given cortisone. H. and E. × 103.

FIG. 3.—Twenty-two hour tuberculin reaction : perivascular infiltration (*a*) in untreated rabbit, (*b*) in rabbit given cortisone. H. and E. × 544.

FIG. 4.—Twenty-two hour tuberculin reaction in untreated rabbit : perivascular infiltration.
(*a*) Stained to show reticulin (Caldwell and Rannie, 1947). × 320.
(*b*) Same blood-vessel. H. and E. × 400.



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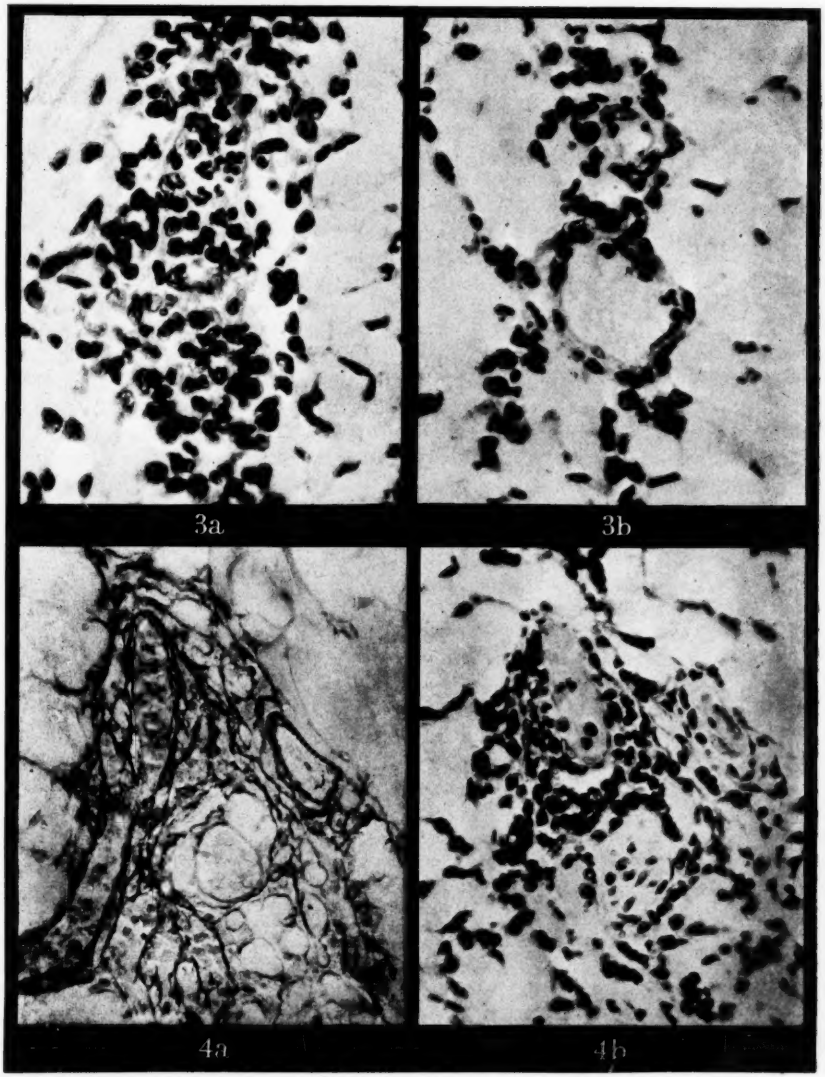
3a
3b
4a
4b

summarised as (i) an inhibition of the vasodilation and, in consequence, of the oedema, features more striking macroscopically than in sections ; (ii) a reduction in the cellular reaction, mainly of the mononuclear cells, but also to a far less degree of the polymorphs ; (iii) an accumulation of degenerate polymorphs in the later stages.

Reaction to Brain Phospholipid.

The injection of brain phospholipid at the concentration used produces an insignificant amount of tissue necrosis and evokes a typical acute inflammatory reaction. A detailed description is therefore unnecessary, and only those points relevant to a comparison with the tuberculin reaction are dealt with.

Macroscopically a moderate swelling is palpable which is in part due to inflammatory oedema (as shown by the serous and serofibrinous exudate), and probably also to a residuum of the relatively poorly diffusible injected material, which before removal during the dehydration process occupies numerous clear spaces between the connective-tissue fibres. By 22 hours the swelling is reduced to a slight papule in the centre of an erythematous area, and the sections show that it is now almost entirely due to inflammatory exudate and cellular infiltration.

4-*hour lesion.*—The cellular infiltration consists almost entirely of polymorphs and is most intense in an irregular region in the compact layer of the dermis, centred round the needle track. From there it spreads out in a rather uniform fashion between the connective-tissue fibres, becoming diffuse and more extensive in the loose areolar tissue layer. The superficial dermis is only involved in so far as it lies within the range of the centrifugal spread of the exciting agent. In the irregular central area polymorphs only are present ; many are degenerate, especially in the dense accumulations mixed with serofibrinous exudate round small necrosed blood vessels. The necrosis involves only the capillary endothelium and few if any of the adjacent fixed tissue cells, and may well be secondary to blocking of the capillaries by leucocytes.

A moderately dense zone of cells surrounds many of the small blood-vessels ; it consists of polymorphs and, in the outer part of the lesion, also of a smaller number of more diffusely distributed mononuclear cells, which resemble the blood monocytes and are presumably of haemic origin. Aggregates of mononuclear cells in close contact with vessel walls are rare and never as large as in the tuberculin reaction, neither is hyperplasia of local histiocytes such a conspicuous feature.

22-*hour lesion.*—The infiltration now has a fine web-like pattern in the compact dermis owing to the dispersion of the injected fluid. Polymorphs are less numerous, many are degenerate and the perivascular infiltrations are less intense. On the other hand, free mononuclear cells have increased in number, though they are still far fewer than the polymorphs and are more uniformly distributed than before : focal accumulations in relation to blood vessels are small and infrequent and not a characteristic feature. A reaction by the local histiocytic cells is now more apparent, but it does not approach that in the tuberculin reaction. The degree of epidermal proliferation is related to the proximity of the underlying lesion, and rarely exceeds 2–3 layers.

To summarise, during the period studied the reaction is dominated by the migration of polymorphs from blood-vessels throughout the area of the lesion towards a central focus, while the local reticulo-endothelial cells play only a minor role.

TABLE I.—*Differential Cell Counts.*

	Four hours after testing.				Twenty-two hours after testing.			
A. *Sub-epidermal infiltration :* Cells present in 10 high-power fields in 10 adjacent interfollicular spaces, in region of maximal reaction.	Mononuclear.	Polymorphonuclear.	Total.	Percentage of mononuclear.	Mononuclear.	Polymorphonuclear.	Total.	Percentage of mononuclear.
Sites of injection of 80 I.U. of old tuberculin	1.				2.			
Normal non-sensitised rabbit . .	74	18	92	(80·4) .	122	44	166	(73·5)
R54. Sensitised rabbit . . .	233	115	348	67·0 .	374	56	430	87·0
R55. ,, ,, . . .	143	70	213	67·1 .	264	49	313	84·3
R57. ,, ,, + cortisone .	140	101	241	58·1 .	210	116	326	64·4
R58. ,, ,, ,, .	127	95	222	57·2 .	110	60	170	64·7
B. *Perivascular infiltration :* Cells present in 20 high-power fields, each centred on a separate blood-vessel.	Mononuclear.	Polymorphonuclear.	Total.	Percentage of mononuclear.	Mononuclear.	Polymorphonuclear.	Total.	Percentage of mononuclear.
Sites of injection of 80 I.U. of old tuberculin	1.				2.			
R54. Sensitised rabbit . . .	578	340	918	63·0 .	1286	272	1558	82·5
R55. ,, ,, . . .	131	87	218	60·1 .	813	216	1029	79·0
R57. ,, ,, + cortisone .	138	192	330	41·8 .	685	466	1151	59·5
R58. ,, ,, ,, .	111	173	284	39·1 .	558	357	915	61·0
Sites of injection of brain-phospholipid 0·2 per cent	3.				4.			
R54. Sensitised rabbit . . .	253	582	835	30·3 .	323	345	668	48·4
R55. ,, ,, . . .	284	624	908	31·3 .	242	293	535	45·2
R57. ,, ,, + cortisone .	74	590	664	11·1 .	91	341	432	21·1
R58. ,, ,, ,, .	70	511	581	12·0 .	77	345	422	18·2

R.54 and R.57 were highly and equally sensitive.
R.55 and R.58 less highly and also equally sensitive.

A simple t test may be applied to the percentages : for example in A_1 :
Mean of untreated animals : $67 \pm 0{\cdot}05$.
Mean of treated animals : $57{\cdot}65 \pm 0{\cdot}45$.

Difference $9{\cdot}35$. Standard error of difference $= \sqrt{0{\cdot}05^2 + 0{\cdot}45^2} = 0{\cdot}45$
$D/SE_D = t = 9{\cdot}35/0{\cdot}45 = 20{\cdot}8$
p (with two degrees of freedom) $= 0{\cdot}01 - 0{\cdot}001$

Similar calculations for all the groups result as follows :

A_1	$t_{[2]} = 20{\cdot}8$	$p = 0{\cdot}1 - 0{\cdot}001$	A_2	$t_{[2]} = 15{\cdot}9$	$p = 0{\cdot}01 - 0{\cdot}001$
B_1	$t_{[2]} = 10{\cdot}7$	$p = 0{\cdot}1 - 0{\cdot}001$	B_2	$t_{[2]} = 10{\cdot}8$	$p = 0{\cdot}01 - 0{\cdot}001$
B_3	$t_{[2]} = 27{\cdot}5$	$p = 0{\cdot}1 - 0{\cdot}001$	B_4	$t_{[2]} = 12{\cdot}6$	$p = 0{\cdot}01 - 0{\cdot}001$

This analysis indicates that the percentage differences between the mononuclear counts in treated and untreated animals are all statistically highly significant.

Effect of Cortisone on Brain Phospholipid Reaction.

The effect of cortisone is much less dramatic than in the case of the tuberculin reaction, and indeed macroscopically there is no significant change. Careful comparison of sections does, however, indicate a slight but definite suppression of the polymorphonuclear infiltration and probably also of the serofibrinous exudate and capillary necrosis. The inhibition of the mononuclear reaction on the other hand is quite definite though, owing to the small number of these cells present in the untreated lesion, it is ineffective in reducing the total intensity of the reaction to any visible degree. As in the tuberculin reaction, reduction in number of the free mononuclear cells appears to be more prominent than any effect on the reaction of local histiocytes. The change effected by cortisone on the proportion of polymorphonuclear and free mononuclear cells around small blood vessels is illustrated by the cell counts in Table I, B, 3 and 4. As might be expected from the absence of any striking reduction of the intensity of the lesion, there is no detectable alteration in the proliferation of the epidermis over it.

DISCUSSION.

Our observations of the histology of the tuberculin reaction in the rabbit suggest that it is essentially a reaction of the local reticulo-endothelial cells, using the term in its broadest sense. Although in its early stages the response bears a superficial resemblance to an acute inflammatory reaction (as exemplified by the reaction to brain phospholipid), histological analysis shows that although the same basic types of tissue response occur in both lesions, their relative proportions, function and development are widely divergent.

1. The response to tuberculin is diffuse, with multiple widely distributed perivascular foci of mononuclear cells. There is no main focus related to the region of initial maximum concentration of the injected material, acting as the centre of attraction for the infiltrating cells, such as is present in the brain phospholipid lesion.

2. The development of each of these perivascular collections of cells is not due to a transient infiltration of cells round small blood-vessels preliminary to their migration elsewhere, nor to the focal accumulation of cells invading the lesion from a number of such blood-vessels, the characteristics in the brain-phospholipid lesion in the early perivascular polymorphonuclear infiltration and of the central cellular zone respectively. It is due rather to the local proliferation of only those mononuclear cells which have migrated from the blood-vessel concerned and of any native reacting cells, and results in relatively durable structures which are to a large extent responsible for the characteristic appearance of the lesion.

3. The diffuse cellular reaction is the result not only of infiltration by cells of haemic origin, but also of hyperplasia and proliferation of indigenous cells of the local reticulo-endothelial system. The situations of these cells and of small blood-vessels are therefore together responsible for the density of the reaction in any one morphologically distinct tissue included in a single lesion. The cellularity and vascularity of the superficial part of the dermis in the rabbit thus accounts for the conspicuous reaction in this layer, and contrasts with the less marked reaction in the underlying compact dermal layer. Further evidence of

the dependence of the response on the tissue substrate is given by the greater intensity of the reaction round vessels in the deep fascia beneath the panniculus carnosus, than round less distant blood-vessels in the dermis proper.

4. Tissue damage and the direct chemotactic activity of the injected material, the fundamental stimuli in an acute inflammatory reaction, play an insignificant role in determining the character and intensity of the cellular response in the tuberculin reaction. Necrosis is absent and centripetal migration of polymorphs is negligible following the injection of tuberculin into control animals, and neither is appreciably greater when sensitised animals are used. Necrosis undoubtedly may occur in an intense tuberculin reaction, and evoke a secondary polymorphonuclear response, but the necrosis coincides with the height of the specific cellular reaction and does not precede it, and may well be a secondary effect due to blockage of small capillaries with leucocytes.

5. New tissue is formed by 22 hours in the tuberculin reaction which may justifiably be called reticulo-endothelial tissue. Additional observations in the guinea-pig show that this persists, without any significant replacement by fibrous tissue, for at least 21 days, and that during this interval cells appear which resemble the transitional cells and plasmablasts described by Fagraeus (1948) in lymph-nodes subjected to repeated stimulation by the specific antigen. This contrasts with an acute inflammatory reaction, the purpose of which is the restoration of normality, and is inconsistent with the description of the tuberculin reaction as an accelerated inflammatory reaction.

Histological analysis therefore suggests that the tuberculin reaction is homologous, not with an acute inflammatory reaction, but with the exaggerated response of lymph-node reticulo-endothelial tissue following a second contact with the specific antigen, modified here by the disposition of the reacting cells in the substrate, i.e., the skin. Viewed in this light comparisons of the relative proportions of the cell types involved and of their time of appearance in the tuberculin and acute inflammatory reaction, which have been the cause of considerable controversy in the past, are irrelevant.

A slight specific polymorphonuclear response which is diffuse and not obviously directed towards any particular centre or centres does, without doubt, occur in the early stages, but this does not invalidate the above analysis of the nature of the tuberculin reaction, since a transient diffuse polymorphonuclear infiltration has been noted in lymph-nodes reacting to antigenic stimulation (Ehrich and Harris, 1942; Marshall and White, 1950).

Our results however show that in spite of the profound difference in the extent to which the tuberculin and brain phospholipid reactions are modified, in both lesions there is a far more marked suppression of the mononuclear cells than of the polymorphs. The apparently conflicting effects of cortisone in the two lesions can therefore be reconciled, without reference to the immune nature of the tuberculin reaction, since the dominant type of reacting cell is the mononuclear in the tuberculin reaction and the polymorph in the other.

The question arises whether any additional specific anti-allergic action of cortisone, or any increased sensitivity to cortisone of cells activated by the immune mechanism, is required to account for the more complete inhibition of the tuberculin reaction. Although our material is insufficient to provide conclusive evidence, the cell counts show that the percentage differences in the mononuclear perivascular infiltrations between treated and untreated animals are approxi-

mately the same in both types of lesion at 4 hours, and are actually greater in the brain phospholipid reactions at 22 hours. If this is indeed the case there is no need to postulate any specific effect of cortisone on the immune mechanism itself: rather it may be assumed that the cellular reaction is suppressed by the same means in both lesions, despite their essentially different structure and function. In line with this is the observation that differences in initial sensitivity do not affect the ratio of mononuclears to polymorphs in the way that cortisone does.

The profound suppression of the hyperaemia in the tuberculin reaction compared with the negligible effect on the brain phospholipid lesion suggests at first sight that the vascular reaction is inhibited to a greater extent by cortisone when the stimulus originates in an immune mechanism than when the stimulus is non-specific. However, in both the vascular reaction is proportional to the degree of cellular infiltration characteristic of each type of lesion, and its modification by cortisone runs parallel with the modification of the accompanying cell reaction. This may reflect an actual dependence of the vascular reaction either on a preceding cellular reaction, or on its specific stimulus, cortisone being more effective in suppressing the stimulus for mononuclear cells than that for polymorphs. This is consistent with an inhibition of both the cellular and vascular reaction by cortisone in the tuberculin reaction which is independent of its allergic nature.

It is of interest that cortisone had no significant effect on the proliferation of the epidermis over either the tuberculin or brain phospholipid lesion; this agrees with findings on human beings (Baxter, Schiller, Whiteside and Straith, 1951), and is unlike the marked suppression of mitotic activity in the epidermis of mice (Green, 1950). In both cortisone-treated and normal control animals the degree of proliferation was related to the intensity and proximity of the underlying reaction.

Our results emphasize the importance of suitable timing when studying the effect of cortisone on the relative proportion of different cell types in tissue reactions. If the tuberculin lesions had only been studied at 22 hours it would have been inferred that cortisone actually increases the polymorphonuclear infiltration (for which the neutrophilia would offer a facile explanation), since they are more numerous at this stage than in the control lesions; but it seems probable that their accumulation is in part due to a delay in mobilisation and in part also to a failure of removal by phagocytosis. Our results may also help to reconcile various opinions of the part played by polymorphs in the early stages of the tuberculin reaction. Dienes and Mallory (1932) state that mononuclear cells predominate in the earliest lesions examined (2 hours) and continue to do so, unless necrosis, which incites a non-specific polymorphonuclear infiltration, occurs. Follis (1940) used P.P.D., and by differential counts in 4-hour lesions found polymorphs to be the most numerous infiltrating cell. We have observed that (1) at 4 hours there is a non-uniform distribution of the polymorphonuclear and mononuclear cells in the various strata of the dermis, so that the results depend on the layer counted, and strictly speaking a true estimate cannot be obtained except by counting the whole of the area involved in the reaction; (2) non-specific reactions due to the solvents used, while they have no significant effect on the gross appearance of the lesion, may provoke a polymorphonuclear response which obscures the specific histological features of the tuberculin reaction. Comparisons between different parts of the lesion and the use of unsuitable

solvents may therefore both be factors in the lack of agreement between different workers.

The striking effect of cortisone in suppressing mesenchymal reactions has been much investigated, but chiefly with reference to long-term effects such as the delayed formation of granulation tissue in healing wounds. Our results show the same mechanism is at work in the modification of acute tissue reactions, both allergic and inflammatory, and are in agreement with the results of Dougherty and Schneebeli (1950) using cell-counts on tissue spreads in mice. The impression that there is less disturbance of the hyperplasia of local tissue cells than of the infiltration of cells from the blood—in other words, that the function of reacting cells is not interfered with provided that they are in the right environment but that the number of cells reaching this position is less—conforms with the consensus of opinion that the modification is quantitative and not qualitative, at least at its inception. Prolonged treatment might well lead to additional qualitative abnormalities. Whether the cortisone-induced lymphopenia and monocytopenia (Aschkenasy, Bussard, Courvazier and Grabar, 1950) is alone responsible for the reduction in infiltrating cells, or whether cortisone also affects the chemotactic stimulus or response, is a problem awaiting investigation. In this connection it is of interest that X-irradiation also both causes a lymphopenia and suppresses tuberculin reactivity.

Modification of the polymorphonuclear cell response by cortisone has until recently been less intensively studied, partly because interest has been focused on the later stages of tissue reactions and partly because the suppression is relatively so slight that it may easily be missed in intense acute lesions. Our observation that there is a less striking but still definite interference with the polymorphonuclear response is quite in line with the work of Dougherty and Schneebeli (1950) and of Michael and Wharton (1951).

We have suggested that the tuberculin reaction is really an attempt to build up, as it were, a local lymph-node at the site of attack in the tissues. Although most current work seems to suggest that tuberculin sensitivity is disadvantageous to the organism, it is possible that (at least in a host less inherently susceptible to tuberculosis than the guinea-pig) multiplication of such patches of reticulo-endothelial tissue at threatened sites might be of value in defence. Cortisone interferes, in a quantitative way, with this process; and clearly this would imply a reduced efficiency in the carrying out of the function for which such an *ad hoc* node is designed.

SUMMARY.

The histological reaction to tuberculin in the skin of sensitised rabbits has been analysed and compared with the reaction to a non-specific irritant (brain phospholipid) at 4 and 22 hours.

The effects of cortisone on each type of lesion have been investigated and contrasted: visual impressions have been confirmed by means of differential cell-counts on sections.

The injection of tuberculin has been shown to result in a diffuse reaction with characteristic multiple perivascular accumulations of mononuclear cells, the result both of cellular infiltration and of local proliferation. Necrosis does not appear to play any part when small doses of tuberculin are used. The resulting tissue is likened to that of a local lymph-node.

In both types of lesion the mononuclear component is markedly reduced by cortisone, the polymorphonuclear component being less affected. The infiltration of cells from outside appears to be more affected than the proliferation of indigenous histiocytes.

Cortisone has a more striking effect on the tuberculin lesion only because this contains a higher proportion of mononuclear cells. Our results give no evidence for a specific anti-allergic activity.

The effects of cortisone on the vascular reaction are described and are shown not to conflict with this analysis.

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THE FIBRINOLYTIC ACTIVITY OF URINE.

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THE fibrinolytic activity of normal urine was first described by Macfarlane and Pilling (1947). The enzyme was found in all samples examined, usually in considerable amounts, and lysis of a fibrin clot was caused by urine dilutions of 1/2000 to 1/4000. Fibrinolytic activity of the circulating blood has also been described in anaphylaxis and peptone shock by Rocha e Silva, Andrade and Teixeira (1946) and by Ungar and Mist (1949). Macfarlane (1937), Macfarlane and Pilling (1946, 1947) and Macfarlane and Biggs (1946) found it in states of anxiety and shock, after exercise, and after the administration of adrenaline. Although exercise produced a slight increase in urinary fibrinolysin the reduction in the rate of urinary excretion made interpretation difficult.

Earlier reports on the presence in urine of proteolytic enzymes in general were made by Farnsworth, Speer and Alt (1946), who showed that the excretion of a pepsin-like enzyme was reduced in pernicious anaemia and other conditions with achlorhydria, and by Baumann and Loeper (1922), who described the excretion of pepsin, erepsin and trypsin-like enzymes and commented on the marked variability in the rate of excretion. The restricted range of pH in which gastric proteinases are active, the highest being 4·5 for cathepsin (Buchs, 1949), excludes the stomach as a source of the fibrinolytic enzyme demonstrated by Macfarlane.

The action of streptokinase on a globulin fraction of plasma (plasminogen) to form an active fibrinolysin (plasmin) was first described by Milstone (1941). The activation of plasminogen by a wide variety of different substances has since been described, including tissue suspensions (Astrup and Permin, 1947, and Lewis and Ferguson, 1950). Tagnon and Petermann (1949) and Tagnon and Palade (1950) showed that rat lung is particularly effective and that the active principle is in the mitochondria. A kinase of the same type has been demonstrated in malignant cells by a number of workers, including Fischer (1946) and Goldhaber, Cornman and Ormsbee (1947).

From this work there would appear to be three possible sources for the urinary fibrinolysin.

The enzyme may be plasmin either excreted after activation in the circulation or as its precursor plasminogen. In this case activation must take place during passage through the renal tract.

Secondly the enzyme may be trypsin which has been absorbed into the blood, combined with serum anti-trypsin and then excreted by the kidneys after separation from its antibody.

Thirdly it is possible that fibrinolysis caused by urine is due to activation of plasminogen contaminating the fibrinogen used as a substrate. The fibrinogen used by Macfarlane (1947) was purified by repeated precipitation, but it is not clear whether complete removal of plasminogen was obtained.

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This paper describes experiments designed to identify the source of urinary fibrinolysin by testing each of these hypotheses.

MATERIALS AND METHODS.

Fibrinogen.

Human fibrinogen was kindly supplied by the Blood Products Research Unit of the Lister Institute. It was used in 0·1 per cent and 0·2 per cent solutions in oxalated phosphate buffer saline, pH 7·6, and was shown to contain plasminogen (*vide infra*).

Thrombin.

Human thrombin was also supplied by the Lister Institute and was used in a buffered saline solution of 5 units/ml.

Estimation of fibrinolysis.

1. *Blood.*—Citrated plasma was examined by the technique described by Macfarlane and Pilling (1946), in which fibrinogen is added to diluted plasma and clotted with thrombin. If lysis of the clot occurred within 18 hours the test was regarded as positive.

2. *Urine.*—All samples of urine were adjusted to pH 7·5 by the addition of strong sodium hydroxide solution, using nitrazine paper as an indicator. Serial 1 : 2 dilutions, volume 0·5 ml., were made in buffered saline down to 1 : 16 and examined by the method described by Rocha e Silva and Rimington (1948) for the estimation of activated ox globulin, by the addition of 0·3 ml. of 0·2 per cent fibrinogen solution. The fibrinogen was clotted by the addition of thrombin and incubated at 37° to allow lysis to occur. Some samples of urine, chiefly concentrated ones, failed to clot unless diluted to 1 : 16. Precipitation of the fibrinogen or antithrombic activity of the urine could both be excluded as possible causes but the phenomenon remained unexplained.

The times required for fibrinolysis by urine varied from 40 minutes for undiluted urine to 24 hours for some 1 : 16 dilutions. The results were recorded as strongly positive if lysis occurred within 4 hours in any tube, positive if lysis occurred overnight in a dilution greater than 1 : 4, and weakly positive if lysis only occurred in a dilution of 1 : 4 or less overnight.

Estimation of proteolysis.

Todd's (1949) modification of the method described by Oakley, Warrack and van Heyningen (1946) was used in which azocoll, a compound between an azo-dye and powdered hide, is employed as the substrate. The colour released from the azocoll was measured in arbitrary colour units in a King single cell photo-electric colorimeter using a green tricolour filter. It was standardized in a series of 25 tests against weighed amounts of crystalline trypsin. The mean readings for each 25 tests were as follows: No trypsin, 20·9 $\pm$ 2·1; 1·6 μg. trypsin/ml., 41·2 $\pm$ 4·4; 3·1 μg. trypsin/ml., 56·8 $\pm$ 2·8; 6·25 μg. trypsin/ml.,80·1 $\pm$ 7·7; and 12·5 μg. trypsin/ml., 108·5 $\pm$ 7·1 colour units. A standard curve was prepared from these figures, the results of proteolysis estimations being expressed in μg. standard trypsin/ml.

Surgical technique.

Rabbits were anaesthetised with 0·5 ml. of veterinary Nembutal intravenously; anaesthesia was maintained with open ether. Excision of the stomach, spleen, pancreas and duodenum was performed through a left paramedian incision. The oesophagus was doubly clamped, ligated and divided, the splenic pedicle ligated and the spleen removed. Clamps were then placed on all vessels from the stomach, duodenum and pancreas. These were ligated and divided in turn. The stomach, duodenum and pancreas were removed in one piece after ligation of the upper end of the jejunum. Surgical procedures on the kidney were carried out through a lateral incision. Injection of the renal artery was performed from the anterior aspect. The nephrectomy was performed from the posterior approach, but the peritoneum was opened and the abdominal contents packed off to provide a reasonable control experiment for the procedures on the stomach.

In these cases the animals chosen were male, and urine was obtained by extraperitoneal suprapubic exposure of the bladder and aspiration with a 10 ml. syringe and a fine needle. Female animals were reserved for experiments involving catheterization.

EXPERIMENTAL.

The Relation of Proteolysis to Fibrinolysis.

The batch of azocoll in use was shown by the standardization experiments to be susceptible to trypsin. Its susceptibility to active plasmin (reported by Todd, 1949) was confirmed by the activation of plasminogen by streptokinase and the demonstration of the release of colour from azocoll by the product (Table V).

The presence of plasminogen in the fibrinogen used as a substrate was shown by the addition of 0·3 ml. streptokinase (Burroughs Wellcome & Co.) in the standard test for fibrinolysis, which occurred in 20 minutes. It was also shown that a solution of trypsin of 1·6 μg./ml. would cause complete fibrinolysis only in 80 minutes. The two reactions thus differ in sensitivity.

In any experiment the demonstration of an enzyme active against azocoll indicates the presence of trypsin, active plasmin or some similar but unidentified active enzyme. The demonstration of fibrinolytic activity must be interpreted however as showing the presence of either an active enzyme such as trypsin or plasmin or of a kinase capable of activating the plasminogen contaminating the substrate. The employment of both tests will thus show the presence of a kinase providing that active plasmin and trypsin are absent or are present in negligible amounts.

The Fibrinolytic Activity of Normal Urine.

Catheter or mid-stream specimens of urine were examined from 53 patients suffering from a variety of diseases and from 13 normal pregnant women. In each case the urine was sterile on culture and contained no excess of leucocytes or red cells in the centrifuged deposit. Fibrinolytic activity was demonstrated in all samples, but no proteolytic activity against azocoll could be found in any of the 22 samples on which the test was carried out.

The effect of variation in the rate of excretion of urine on its fibrinolytic activity was shown by collecting all urine from a normal subject at intervals, including an overnight sample, and during a diuresis induced by drinking two pints of water. The results of this experiment are shown in Table I.

TABLE I.—*The Fibrinolytic Activity of Urine from a Normal Subject.*

Time between specimens in minutes.	Volume of specimens (ml.).	Rate of excretion (ml./min.).	Time for fibrinolysis of 1 : 2 dilution (min.).
630	385	0·6	100
120	135	1·1	80
50	52	1·0	100
100	122	1·2	100
20	200	10·0	170
10	115	11·5	180
5	59	11·8	180
5	62	12·4	170

The figures show a decrease in the fibrinolytic activity of the urine with an increase in its rate of excretion. They also show that over a short period of time a water diuresis will cause a constant rate of excretion between 10 and 13 ml./minute. It is possible therefore to study the factors which influence the fibrinolytic activity of normal urine provided that a diuresis is induced and samples are taken at short intervals.

The Effect of Active Plasmin in the Blood on the Excretion of Fibrinolysin.

Man.

Water was given to normal subjects and samples of urine collected until a constant rate of excretion was established. Adrenaline hydrochloride 1 : 1000 was injected intramuscularly in a dose of 0·5 ml., and then repeated in doses of 0·2 ml. until either tachycardia, an increase in the systolic blood pressure, a marked change in the reflexes or a coarse tremor occurred. Blood was taken by venepuncture and samples of urine collected at intervals during and until 30 minutes after the injection. The blood and urine were examined for fibrinolytic activity, the urine also being examined by the azocoll technique.

In three such experiments the injection of adrenaline caused a reduction in the rate of excretion of urine, but in eight others no such change occurred. In all cases the blood showed active fibrinolysis, but no increase in the activity of the urine was observed. The results of a typical experiment are shown in Table II.

TABLE II.—*The Fibrinolytic and Proteolytic Activity of Urine after the Administration of Adrenaline in Man.*

Time in minutes.	Blood pressure (mm. Hg.).	Injection of adrenaline (ml.).	Rate of excretion of urine (ml./min.).	Fibrinolysis : urine.	Proteolysis (trypsin (μg./ml.).
0	130/78	—	—	Strongly positive	Zero
11	—	—	11·2	Positive	,,
24	160/74	1·1	10·0	,,	,,
35	138/80	—	10·1	,,	,,
60	—	—	12·0	,,	,,

Blood was taken six minutes after the injection of adrenaline and was found to show active fibrinolysis. The azocoll control tube gave a reading of 19 colour units.

Rabbit.

The examination of catheter specimens of urine from six rabbits showed marked fibrinolytic activity with no significant degree of proteolysis of azocoll.

Thus in the rabbit the same disparity of proteolytic and fibrinolytic activity has been observed as was seen in man. It was hoped to induce fibrinolytic activity in the blood of rabbits by the injection of adrenaline in order to repeat the experiments described above in man, but increasing doses, including one fatal one, caused no fibrinolysis in blood removed from an ear vein five minutes after the injections. Previous workers, including Tillett and Garner (1933) and Kaplan (1946), have commented on the resistance of rabbit plasma to fibrinolysis.

The Influence of Alterations of the Gastro-intestinal Secretion on the Excretion of Fibrinolysin.

Man.

Under the controlled conditions used for the experiments with adrenaline the effect of the administration of histamine, prostigmine and carbachol was investigated. Histamine was given in doses up to 1·0 mg. by subcutaneous injection with the production of headache and flushing, but with no influence on the excretion of fibrinolysin in any of seven experiments.

Prostigmine was given in a dose of 0·1 mg. and carbachol in a dose of 0·25 mg. each on two occasions also with no effect on the excretion of fibrinolysin in the urine.

The urine from 13 patients who had undergone subtotal gastrectomy a few days previously was examined, and compared with urine from 12 patients in the same wards who had experienced a surgical operation not on the gastro-intestinal tract. Both series showed a high and similar degree of fibrinolytic activity.

Rabbit.

Since rabbit urine shows marked fibrinolytic activity but no significant degree of proteolysis of azocoll this animal was chosen to determine the effect of the intravenous injection of trypsin on the excretion of fibrinolysin. Crystalline bovine trypsin (Armour & Co.) was dissolved in sterile saline and used for the experiments. To establish the validity of the conclusions toxicity tests were carried out by which it was shown that in rabbits doses up to 1·0 mg. intravenously produced no visible effect. A dose of 10 mg. given to one animal was rapidly fatal.

Rabbits were catheterised and samples of urine taken before and at intervals after the injection of 1·0 mg. trypsin dissolved in 0·2 ml. saline. The samples of urine were examined for fibrinolytic and proteolytic activity. The results of one of five such experiments are shown in Table III, the rate of excretion of urine remaining reasonably constant throughout.

TABLE III.—*The Fibrinolytic and Proteolytic Activity of Rabbit Urine after the Intravenous Injection of Bovine Trypsin.*

Time in minutes.	Injection of trypsin.	Fibrinolysis.	Proteolysis (trypsin μg./ml.).
0	1·0 mg. at 6 min.	Positive	Zero
14	—	,,	1·8
30	—	,,	3·0
72	—	,,	1·3

Azocoll control tube = 25 colour units.

The table shows a marked increase in the proteolytic activity of the urine within a few minutes of the injection and a decrease after 30 minutes. Direct injection into the renal artery of 2·0 mg. trypsin also produced an increase in the proteolytic activity of the urine (from zero to 1·5 μg. trypsin/ml.), but was associated with a marked reduction in the rate of excretion of urine presumably due to cortical arterial spasm and shock.

Attempts were made to influence the rate of excretion of fibrinolysin by total gastrectomy accompanied by excision of the pancreas, duodenum and spleen in rabbits. Three experiments of this type were performed. The animals were given intravenous saline at intervals during and after the operations, and were maintained under light anaesthesia until they were killed a few hours later. As a control a right nephrectomy was performed on another animal, which was allowed to survive. Samples of urine taken from these animals before, during and after the operation were examined. The results of one of these experiments and of the nephrectomy are shown in Table IV.

TABLE IV.—*The Fibrinolytic and Proteolytic Activity of Rabbit Urine after Total Gastrectomy with Excision of Pancreas, Spleen and Duodenum and after Nephrectomy.*

Rabbit.		Fibrinolysis.	Proteolysis (trypsin μg./ml.).
210/50	Before operation	Strongly positive	Zero
	At end of total gastrectomy (45 min.)	„ „	0·8
	After further 90 min.	„ „	1·2
208/50	Before operation	Positive	Zero
	At end of nephrectomy (30 min.)	„	„
	After further 60 min.	„	„

Azocoll control tube = 28 colour units.

These experiments show no appreciable change in the excretion of fibrinolysin after removal of the source of gastro-intestinal enzymes within two hours of clamping the venous drainage of the pancreas and duodenum. Since injected trypsin is mostly excreted within 30 minutes it is believed that if the fibrinolysin were derived from the pancreas a definite fall in activity of the urine would have been found in two hours.

Activation of Plasminogen by Urine in Man.

Plasma was added to urine and incubated for 15 minutes at 37° and examined for fibrinolytic and proteolytic activity. The findings were compared with the activity of plasma and urine alone and with plasma after activation with streptokinase. The results of one of several such experiments are shown in Table V.

TABLE V.—*The Activation of Human Plasminogen by Urine and Streptokinase.*

Urine (ml.).	Plasma (ml.).	Streptokinase (ml.).	Saline (ml.).	Fibrinolysis.	Proteolysis (trypsin μg./ml.).
0·5	—	—	0·5	Weakly positive	Zero
0·5	0·5	—	—	Strongly positive	4·6
—	0·5	0·5	—	„ „	5·2
—	0·5	—	0·5	Weakly positive	3·0
—	—	—	1·0	Negative	Zero

The citrated plasma used in these experiments was obtained from normal subjects, and showed slight spontaneous fibrinolysis with a moderate degree of activity against azocoll. The figures for proteolysis of plasma activated by urine or streptokinase are greater than for plasma alone, and this is supported by the marked difference in the degree of fibrinolysis. The presence of two sources of fibrinogen (in the tests for fibrinolysis only) complicates interpretation, but in fact the greater amount of substrate conceals an even higher fibrinolytic activity than is apparent.

DISCUSSION.

The experiments described above show that in both man and the rabbit the fibrinolytic activity demonstrable in urine is not accompanied by an equivalent degree of proteolytic activity, the level of the latter being too low to be detectable by the azocoll technique. Plasmin has been shown to be active against azocoll; hence it appears unlikely that the fibrinolysin in urine is active plasmin. It has also been shown that as far as the response to adrenaline is concerned the appearance of plasmin in the blood is not followed by a change of the level of the fibrinolytic activity in the urine.

It has further been shown that the intravenous injection of trypsin in rabbits leads to its appearance in the urine in amounts detectable by the azocoll method without an appreciable change in the fibrinolytic activity. Neither subtotal gastrectomy in man nor resection of the stomach, spleen and duodenum in the rabbit leads to a reduction in the fibrinolytic activity of the urine. It appears therefore that urinary fibrinolysin is unlikely to be derived from the proteolytic enzymes of the gastro-intestinal tract.

The presence of plasminogen in the fibrinogen used as a substrate was demonstrated by its activation when streptokinase was added. It appeared possible therefore that the fibrinolysis induced by urine was due to the activation of plasminogen by a kinase present in the urine, a hypothesis which was supported by direct experiment. It seems probable that the activating substance is derived from the renal tract.

SUMMARY.

The fibrinolytic activity of urine is confirmed in man and the rabbit.

Activation of the circulating plasminogen does not alter the rate of fibrinolysin excretion in the urine.

Urinary fibrinolysin does not appear to be derived from the gastro-intestinal tract.

The fibrinolytic activity of urine seems to be due to a kinase which is capable of activating plasminogen. The kinase is probably derived from the tissues of the renal tract and activates plasminogen which is present as a contaminant in the fibrinogen substrate.

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THE PRESENCE OF THIOL GROUPS IN THROMBOCYTES AND THEIR SIGNIFICANCE IN THE CONTRACTION OF FIBRIN GEL.*

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THE contraction of a blood-clot can occur *in vivo*, and has been observed in shed blood when it is collected in containers which have no adhesive affinity for fibrin (Hirschboeck, 1940). Opinions are divided regarding the mechanism of the shrinkage of fibrin. It may be due to synaeresis, a process which according to Ostwald (quoted from Alexander, 1946) is one of the most characteristic properties of a gel, and this could be the result of desolvation of polar groups. According to another view the thrombocytes are implicated in the retraction of fibrin. There is no doubt that the thrombocytes do influence this process. A parallelism between thrombocyte concentration and intensity of clot retraction has been recognized by Denys (1887), Hayem (1896), and it has been confirmed by many workers, including Tocantins (1934, 1938) and Quick, Shanberge and Stefanini (1949). The mechanism of this phenomenon is not understood. Budtz-Olsen (1951) has summarized and amplified the supposition of several earlier workers that some vital action of thrombocytes causes fibrin gel contraction. In order to learn whether any specific chemical groupings are responsible for the activity, the following experiments were carried out and the results are reported.

EXPERIMENTAL.

Human blood was obtained from normal donors by venepuncture. Rabbit blood was taken from the marginal ear vein, and guinea-pig blood was obtained by heart puncture of animals anaesthetized with ether. Blood was mixed quickly with one-ninth of its volume with 0·13 M trisodium citrate solution. The thrombocytes were separated from the other cells by centrifugation at approximately 180 **g** at + 2° for 20 minutes. In order to remove erythrocytes the plasma layer was subjected to the same procedure.

The supernatant fluid with thrombocytes was centrifuged at 2300 **g** for 20 minutes. The sediment was suspended in a chilled solution containing 0·026 M trisodium citrate and 0·12 M sodium chloride. Centrifugations and washings were repeated until the plasma proteins were removed. The thrombocytes were finally suspended either in citrate-sodium chloride or in veronal buffer of Michaelis (1931). A μ of 0·154 was maintained. Counting of the thrombocytes was carried out by the technique of Rees and Ecker (1923). Their purity and integrity were assessed by Leishman staining. The thrombin preparations

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used were a commercial product, beef thrombin topical (Parke Davis), and one made from human plasma essentially according to the technique of Eagle (1935). All the materials were kept in ice water. Plasma specimens and thrombocytes were used on the day of collection and preparation. Fibrinogen was prepared from human plasma by three different procedures. The first, obtained as a dried product isolated by precipitation with ether at $-5°$ from citrated plasma according to the process of Kekwick, MacKay and Record (1946), was shipped from London (preparation A in Table IV). The second preparation was made from oxalated plasma after removal of prothrombin by adsorption on barium sulphate suspension. Fibrinogen was separated by three successive precipitations with 25 per cent saturated ammonium sulphate. Ammonium sulphate was removed by dialysis against a solution containing 0·02 M sodium oxalate and 0·12 M sodium chloride (preparation B in Table IV). The third product was obtained from citrated plasma by precipitation with 1·2 volume of saturated sodium chloride and the residue was washed with 0·5 saturated sodium chloride solution (preparation C in Table IV). These procedures were carried out between 2 and 5°. The purity of the fibrinogen preparations was determined by the ratio of clottable to total protein, using the biuret reaction as modified by Robinson and Hogden (1940). (The clotting and contraction experiments were carried out in soft glass tubes (1 × 7·5 cm.). The temperature of the thermostats varied not more than $\pm$ 0·5°. Contraction of fibrin depends upon several factors. Lundsteen (1942) found optimal conditions at a fibrinogen concentration of 0·1 per cent, a fact which has been confirmed by later workers. The influence of thrombin concentration on fibrin shrinkage has been studied by Quick (1950), and it has been confirmed that higher thrombin concentrations are more effective than lower ones. A pH between 6·0 and 8·2 and temperature between 27° and 37° have been found of little influence by Ellicott and Conley (1951). In setting up the experiments all these factors were taken into consideration. Thrombocyte suspensions from several donors were pooled and preparations containing between 1·0 and 3·5 × 10^6 thrombocytes/c.mm. were employed. Mixtures of 0·25 ml. of the suspension and 0·1 ml. of the reagent were incubated for periods between 5 and 30 minutes at 37·5°, or in some cases at 27·5°. The reagents were applied in a concentration which would not interfere with fibrin formation. To this mixture was then added 0·4 ml. of citrated human plasma freed of thrombocytes by centrifugation. After one minute at 37·5° 0·25 ml. of thrombin dissolved in veronal buffer, pH 7 to 7·4, was added. The clotting times were adjusted to 15–20 seconds. After thorough mixing of the contents the tubes were left undisturbed for 20 hours. One hundred per cent contraction means that a maximum volume of fluid (0·98 ml.) was exuded from the fibrin gel. The fluid was collected by gentle tilting of the tube. In Table I results of experiments are given in which drugs with an inhibitory action on the contraction of fibrin gels were used.

The number of thrombocytes in the reaction mixtures varied from 2·5 to 8 × 10^5/c.mm. This is far in excess of the minimum effective concentration, since under the above conditions 4×10^4 thrombocytes/c.mm. will give 100 per cent fibrin retraction. The figures in Table I are calculated for 10^6 thrombocytes/c.mm.

These results show that mercury ions were the most effective inhibitors. In order to avoid the thermal inactivation of thrombocytes which frequently occurs

TABLE I.—*Reagents Effective in Inhibiting Fibrin Gel Contraction.*

Reagent.	Lowest concentration producing 100 per cent inhibition.
Mercuric chloride	$2 \cdot 5 \times 10^{-5}$ M
p-chloromercuro benzoic acid	10^{-4} M
Sodium iodoacetate	10^{-4} M
Cupric sulphate	$2 \cdot 5 \times 10^{-4}$ M
m-amino-*p*-hydroxy phenylarsenoxide	$3 \cdot 0 \times 10^{-4}$ M
Ninhydrin	5×10^{-4} M
Sodium arsenite	$6 \cdot 6 \times 10^{-4}$ M
Di-(2 chlorethyl) methylamine hydrochloride	10^{-3} M
Formaldehyde	$1 \cdot 5 \times 10^{-3}$ M
Hydroquinone	$6 \cdot 6 \times 10^{-3}$ M

on incubation at 37·5° for 30 minutes, several inhibitors were also used at 27·5°. The temperature coefficient for the thrombin fibrinogen reaction Q_{10} (27·5°–37·5°) is very low, approximately 1·1. Thus it was possible to use the same thrombin concentration in both series of experiments. The results obtained at the two temperatures were similar within the limits of experimental error, indicating that the effects produced were entirely due to the reagents. No appreciable difference in the inhibition by mercury compounds was observed between thrombocytes suspended in solutions of sodium citrate-sodium chloride or veronal buffer. Cupric ions are approximately 10 times less effective than mercury ions, and in contrast to the latter cupric ions in the presence of citrate are less inhibitory presumably because of complex formation. Two preparations of trivalent arsenic compounds were used. It was found that $6 \cdot 6 \times 10^{-4}$ M inorganic arsenite inactivated 10^6 thrombocytes/c.mm. : *m*-amino-*p*-hydroxy phenylarsenoxide was an effective inhibitor in a concentration of 3×10^{-4} M. Another mercurial—mersalyl—was also tested. In concentrations up to 10^{-3} M it did not inhibit fibrin retraction. The effect of the other inhibitors is discussed later.

The reversal of the inhibitory effect of mercury ions on fibrin retraction was attempted. When thrombocytes were incubated with 10^{-4} M mercuric chloride at 37° for 5 minutes, 10^{-3} M cysteine was unable to reverse the inhibition. However, the mercury effect could be annulled by the simultaneous addition of mercuric chloride and cysteine.

In addition to the quantitative tests the nitroprusside reaction was carried out as modified by Hammett and Chapman (1938): 1 ml. of preparations containing 2·1 and $3 \cdot 5 \times 10^6$ thrombocytes per c.mm. gave distinct pink colours. When the thrombocytes were incubated with 2×10^{-4} M mercuric chloride for five minutes at 37° the nitroprusside test became negative. Thrombocytes treated with mercuric chloride or ninhydrin which are unable to induce fibrin retraction were tested for their thromboplastic properties.

The experiments set out in Table II were carried out in the following manner: Column I: 0·4 ml. of citrated cell-free human plasma and 0·35 ml. veronal buffer pH 7·0 were incubated at 37° for 5 minutes, and 0·25 ml. 0·05 M calcium chloride was added. For the experiments in column II the veronal buffer was replaced by 0·25 ml. thrombocyte suspension and 0·1 ml. veronal buffer. For the experiments in column III the 0·1 ml. of veronal buffer in column II was

TABLE II.—*Influence of Thrombocytes on Plasma Coagulation Time and Fibrin Contraction.*

(Coagulation times in seconds.)

Human citrated plasma.	I. Plasma free of thrombocytes.	II. Plasma + intact thrombocytes.	III. Plasma + treated thrombocytes.
1	295	150	148
2	210	125	125
3	378	180	200
4	215	85	90
5	300	100	100
6	230	90	88
7	230	105	105
8	195	90	100
9	225	125	145
Fibrin contraction	*nil*	100 p.c.	*nil*

replaced in experiments 1–6 by $3{\cdot}3 \times 10^{-4}$ M mercuric chloride, and in experiments 7–9 by 10^{-2} M ninhydrin.

Despite the fact that the mercury- or ninhydrin-treated thrombocytes inhibited the contraction of fibrin, their ability to shorten plasma coagulation time was not diminished. In further experiments the following reagents were found not to inhibit fibrin gel contraction : 10^{-2} M potassium ferricyanide, 10^{-2} M sodium tetrathionate, 10^{-2} M sodium cyanide, 10^{-1} M maleic acid, 10^{-1} M ethyl alcohol. Higher concentrations could not be used owing to their interference with fibrin formation.

It is apparent that substances which are commonly considered to react with thiol groups can be divided into two categories with regard to their ability to react with thrombocytes. Inhibitory effects are produced by mercaptide-forming compounds (mercurials, copper ions, arsenicals), by alkylating substances (iodoacetate and nitrogen mustard) and by an oxidizing reagent (ninhydrin), and a very weak effect was obtained with hydroquinone. Negative results were obtained with oxidizing substances (ferricyanide and tetrathionate) and with cyanide, maleate and ethyl alcohol. The great variation in reactivity of the various compounds towards thrombocytes was thought to be due partly to a reversible reaction between the drugs, the active centres of the thrombocytes and similar groups in the plasma proteins accompanying fibrinogen. Such a consideration could be applied to formaldehyde. In order to test this assumption, fibrinogen was separated from human plasma and clot retraction studies carried out with suitable combinations of the isolated components. Experimental conditions were those described for the results in Table I. The results are shown in Table III.

The fibrinogen preparations were diluted with veronal buffer pH 7·0 to 7·4 to give a final concentration of 80–100 mg. per cent in the reaction mixture. Preparation A contained 92 per cent fibrinogen. It was 1 year old at the time of use. During transport by boat no precautions were taken with regard to storage. Preparation B had a purity varying from 88 to 93 per cent fibrinogen. The fibrinogen concentration of preparation C was 60 per cent of the total protein

TABLE III.—*The Influence of Fibrinogen and other Plasma Components on Fibrin Gel Retraction.*

I. Fibrinogen preparation.	II. Additions in ml.	III. Fibrin gel retraction in per cent.
A	*nil*	5–35
,,	0·1 H.D.P.	50
,,	0·05–0·2 H.S.	50–100
,,	0·1 H.S. Cit.	90
,,	0·10–0·3 H.S. heated	100
,,	0·05–0·2 R.S.	70–90
,,	0·05–0·1 G.P.S.	60–80
B	*nil*	0
,,	0·05–0·2 H.S.	65–90
,,	0·10–0·2 G.P.S.	80–90
C	*nil*	0–20
,,	0·2 H.S.	100

Abbreviations in column II: H.D.P. = human citrated plasma defibrinated by thrombin. H.S. = human serum 4 hours old. H.S. cit. = human serum + 0·2 vol. 3·8 per cent trisodium citrate. H.S. heated = human serum heated to 55° for 30 min. G.P.S. = guinea-pig serum. R.S. = rabbit serum. The number of thrombocytes in the reaction mixture varied from 1·4 to 3 × 10^6 thrombocytes/c.mm.

concentration. The experiments were carried out with thrombin preparations of different origin and strength. It was considered possible that preparation A could be defective in certain properties, despite the fact that the thrombin clotting time at a 100 mg. per cent level was similar to that obtained with citrated human plasma specimens. Consequently preparation B, not older than 2 days, was used, and finally preparation C was used 3 to 4 hours after collection. Since similar results were observed with the various fibrinogen preparations it is obvious that clotting of isolated fibrinogen by thrombin in the presence of thrombocytes is not followed by appreciable fibrin contraction. Additional factors are required to complete this process. As can be seen from experimental results given in column II, Table III, these factors are present in plasma and serum of humans, rabbits and guinea-pigs. They are still active after 30 min. heating to 55°. The optimal quantity is present in volumes between 0·1 and 0·2 ml.

DISCUSSION.

Le Sourd and Pagniez (1913) have indicated that the integrity of thrombocytes is essential for their activity in fibrin retraction. Even such mild procedures as freezing and thawing lead to complete loss of this property, as has been shown by Werner (1943). Apparently for effective fibrin retraction the active centres of the thrombocytes must have a particular spatial arrangement. Therefore greater caution is necessary in the interpretation of results obtained with thrombocytes treated with chemicals than when dealing with soluble substances and colloids. Further it is recognized that most of the reagents are not specific for a particular chemical grouping; e.g., mercury compounds react

preferably with thiol groups, but other radicles present in amino-acids and proteins can combine with mercury, as has been shown by Vickery and Gordon (1933) and by Salle and Ginoza (1943). The same applies to iodoacetate, which has been found by Dickens (1933) to block thiol groups, but Michaelis and Schubert (1937) observed that a slower reaction can also take place with amino groups.

In spite of these limitations it would appear that reagents commonly employed for the detection of thiol groups inactivate thrombocytes with regard to their property to contract fibrin gel. On the other hand certain compounds such as potassium ferricyanide and sodium tetrathionate, reagents which oxidize simpler thiol compounds readily, were up to 0·01 M concentration without effect on thrombocytes. However, that is not uncommon with enzymes ; e.g., β-amylases are considered to require thiol groups for full activity, and yet Weill and Caldwell (1945) and Englard *et al.* (1950) observed that 10^{-3} M ferricyanide had no influence whilst 10^{-5} M mercuric chloride produced 100 per cent inactivation. The reaction with ferricyanide and tetrathionate requires two thiol groups, and the non-reactivity of these reagents with thrombocytes suggests steric hindrance, or perhaps the inactivity is due to the fact that the reagents are water-soluble and unable to penetrate the lipoid layer of the thrombocytes, whilst lipoid-soluble materials (mercuric chloride) would be effective. Inactivation of the thrombocytes was obtained at comparatively low concentrations of the reagents, and was not markedly altered by temperature changes between 27° and 37°. The results suggest that the active centres of the thrombocytes essential for fibrin contraction are thiol groups. Complete evidence for this assumption would require reversibility of the inhibition by another substance containing thiol groups of greater affinity towards the inhibitors than the thrombocytes. However, we have only been partly successful in counteracting the inactivation. Supporting evidence for the presence of thiol groups in the thrombocytes has been given by the positive nitroprusside reaction, which was abolished by exposure of the thrombocytes to mercuric chloride.

Ninhydrin and formaldehyde were also found to be inhibitors. These substances are usually considered to react with amino groups. Ninhydrin was tested because Chargaff and Ziff (1941) and Chargaff and Bendich (1943) found that it belongs to a group of compounds capable of converting fibrinogen into a fibrin-like material in the absence of thrombin, and it would have been interesting to see whether such material would contract as does fibrin produced by thrombin. However, in agreement with Ferguson and Ralph (1942) no gelling of citrated human plasma by ninhydrin could be observed. In some experiments a shortening of the thrombin clotting time was noticed. This indicates that ninhydrin could be classified as a fibrinoplastic substance as defined by Ferguson (1940). Of greater interest in this discussion is the influence of ninhydrin on the thrombocytes. Ninhydrin is approximately 20 times less effective than mercuric chloride. In addition to reactivity with amino groups Olcott and Fraenkel-Conrat (1947) state that they have evidence that ninhydrin also oxidises thiol and possibly other groups. Assuming a reaction with thiol groups of thrombocytes ninhydrin would be the only compound tested to have the ability to produce a disulphide linkage, since oxidation of the hydrogen of a thiol and that of a different radicle is not very probable. Formaldehyde as an inhibitor was a hundred times less effective than mercury ions. Although

formaldehyde reacts with amino groups, it has been shown by Anson (1941) that thiol radicles can be blocked by formaldehyde.

On the basis of the above results it is therefore possible that in addition to thiol groups other radicles present in the thrombocytes are involved in the process of fibrin retraction.

Inactivity of cyanide in 0·01 M concentration may be taken as evidence that -S-S-groups are not present in the thrombocytes or are not required for fibrin retraction. It should be pointed out that with the exception of mercury ions the concentrations of inhibitors found to be effective represent minimal values. Especially in the case of formaldehyde it was thought that the comparatively low potency of this substance was due to a reversible reaction between active centres of the thrombocytes and the plasma proteins accompanying fibrinogen.

Experiments with the isolated fractions indicated that fibrinogen, thrombin and thrombocytes are insufficient for complete fibrin contraction; additional components are required. It is suggested that such principles be called contraction co-factors. The finding that fibrin gel retraction does not take place unless other colloids are added to the system has been made recently by Ellicott and Conley (1951). We are in agreement with their findings, except that under our experimental conditions greater quantities of the colloids had to be added to complete gel contraction. The probable explanation for this quantitative difference is that Ellicott and Conley were working with silicone-coated tubes, which show less adhesiveness to the fibrin gel than the soft glass tubes used in this work, and consequently their procedure requires smaller amounts of colloid to overcome the adhesive forces attracting the fibrin gel. The various fibrinogen preparations show the same behaviour with regard to clot retraction, indicating that the isolation procedure and storage had little effect on the retraction of the fibrin gel and we were not dealing with deficient fibrin preparations. With the reported findings one more instance of a biological system requiring thiol groups for activity has been added to the list. The occurrence of thiol groups in cell fluids was established by the discovery of glutathione by Hopkins (1921), and the observation of Rapkine (1930) that cell division and growth are correlated with thiol activity indicated the ubiquitous biological importance of thiol groups. They are present in bacteria (Fildes, 1940). The connection between thiol groups and enzymic activity has been summarized by Singer and Barron (1945) and by Gordon and Quastel (1948). Stetson and Thomas (1948) reported an influence of thiol groups on the sickling of red cells, and McLeod (1951) found that thiol groups are essential for motility of spermatozoa. Mazur *et al.* (1950) have shown that the vasodepressor activity of ferritin is dependent on free thiol groups. Perhaps of some interest in this connection is the fact that gelation of pectin appears to depend upon the presence of thiol groups. Kopaczewski (1949) and Huggins *et al.* (1951) and Wormell (1951) report that certain proteins can only be gelled when thiol groups are available. Finally, it is noteworthy that Buchthal, Deutsch, Knappeis and Munch-Petersen (1947) and Bailey and Perry (1947) found that thiol groups are required for the synaeresis of actomyosin fibres in the presence of adenylpyrophosphate.

SUMMARY.

The physiological contraction of fibrin gel depends upon the presence of certain chemical groups in intact thrombocytes. These can be blocked by a

variety of reagents commonly considered to combine with sulphydryl radicles. It is probable that other groups are also involved in this process. In addition to fibrin, thrombin and thrombocytes, certain heat-stable colloids present in defibrinated plasma or serum are required for complete fibrin contraction. These compounds have been termed contraction co-factors. They are not species specific. The fact that additional colloids are required for fibrin retraction is a confirmation of other recent observations.

Thrombocytes treated with mercuric chloride or ninhydrin which are inactive in fibrin contraction show still undiminished thromboplastic potency.

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THE ACTION OF ACRIDINE COMPOUNDS ON CELL DIVISION.

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DUSTIN (1947) has described the histological changes produced by trypaflavine in dividing cells and considers them similar to those produced by ionising radiations. He therefore includes trypaflavine with urethane and nitrogen mustard in a " radiomimetic " group of mitotic poisons. Loveless and Revell (1949), however, consider that all three substances inhibit cell division by affecting general cellular processes and thus differ in their mode of action from ionising radiations.

Trypaflavine is one of the acridine group of compounds, some of which are antimalarial and some antibacterial (see Albert, Rubbo, Goldacre, Davey and Stone, 1945), while other compounds restrain the growth of experimental tumours in mice (Lewis and Goland, 1948). It is possible that all these effects may be due to mitotic inhibition, for this has been demonstrated in a series of acridine compounds by Lasnitzki and Wilkinson (1948) using cultures of chick fibroblasts. Inhibition of cell division by acridine compounds may be due to interference with carbohydrate metabolism, for atebrin has been shown to inhibit carbohydrate metabolism by effects on glucose-6-phosphate dehydrogenase (Haas, 1944) and on hexokinase (Speck and Evans, 1945). Such an inhibition of carbohydrate metabolism may cause inhibition of cell division because of the association between the two processes, which has been demonstrated in the mouse epidermis (Bullough, 1950) and in the developing midbrain and red blood cells of the embryonic chick (O'Connor, 1950*a*, 1950*b*, 1951).

To examine this possibility that acridine derivatives affect cell division by modifying carbohydrate metabolism investigations have been made on the isolated midbrain of the chicken embryo. In this tissue the minimum concentrations of a series of acridine compounds causing mitotic damage have been determined, together with the effects of the compounds on carbohydrate metabolism.

METHODS.

Material.

In all experiments the midbrain of the 6-day chicken embryo was used, the dorsal half being removed and placed in one of the media to be described. The external coverings were then removed and the midbrain cut in fragments 1–2 mm. square for use in the experiments. Four acridine compounds were investigated and these are listed in Table I. The first three compounds were obtained from Dr. J. H. Wilkinson and the fourth from Professor A. Albert, to both of whom gratitude is expressed.

TABLE I.—*The Effective Mitotic Concentration (E.M.C.) of Acridine Compounds in the Isolated Midbrain of the 6-day Chicken Embryo.*

Compound.	E.M.C. in medium containing phosphate buffer M × 10^{-4}.	E.M.C. in medium containing bicarbonate M × 10^{-4}.
5-Aminoacridine hydrochloride	0·32	0·40
5-Amino-2-trifluoromethylacridine hydrochloride	0·32	0·50
5-Amino-1-phenyl-10-methylacridinium bromide	0·50	0·61
5-*Cyclo*hexylaminoacridine hydrochloride	0·40	0·33

Estimation of mitotic inhibition.

For each of the acridine compounds of Table I the concentration was determined which, in the isolated midbrain, caused fusion of the separated chromosomes in dividing cells. This change resembles that described by Dustin (1947) as pycnosis and has been described previously (O'Connor, 1949). To determine this "effective mitotic concentration" (E.M.C.) the technique previously described was used (O'Connor, 1950*b*). Portions of the isolated midbrain were exposed to graded concentrations of the acridine compounds for 90 min. at 37° C. For each acridine compound the E.M.C. was determined under two sets of conditions: firstly in a medium containing phosphate buffer in an atmosphere of oxygen; and secondly, in a medium containing bicarbonate in an atmosphere of 95 per cent oxygen plus 5 per cent carbon dioxide. These conditions corresponded to the conditions in which changes of respiration and aerobic glycolysis respectively were measured (see below).

Investigation of carbohydrate metabolism.

In the first week of incubation the source of energy in the developing chick is predominantly carbohydrate (Needham, 1926). Hence it may be assumed that the previous demonstration of a respiratory quotient of unity in the midbrain of the 6-day embryo (O'Connor, 1950*a*) indicates that the oxygen consumed in respiration is used for the metabolism of carbohydrate. Alterations in carbohydrate metabolism may therefore be shown by alterations in the rate of respiration or in the respiratory quotient. The effect of the acridine compounds on both has been measured. In addition, in the presence of oxygen, carbohydrate is broken down by aerobic glycolysis; the effect of the acridine compounds upon the rate of this has also been measured. The measurements were made by a modification of the Cartesian Diver micromanometer using techniques previously described (O'Connor, 1948, 1949, 1950*a*, 1950*c*). In these experiments the following media were used:

(*a*) For respiration and respiratory quotient: NaCl 0·90 g., KCl 0·02 g., $MgCl_2$ 0·02 g., $CaCl_2$ 0·02 g., glucose 0·10 g., water 100 ml. To this was added 10 c.c. of Sørensen's M/15 phosphate buffer to produce pH 7·4. The atmosphere in the divers was oxygen.

(*b*) For aerobic glycolysis: NaCl 0·90 g., KCl 0·02 g., $MgCl_2$ 0·02 g., $CaCl_2$ 0·02 g., $NaHCO_3$ 0·20 g., glucose 0·10 g., water 100 ml. The atmosphere in the divers was 95 per cent oxygen plus 5 per cent carbon dioxide. In both media the weights given refer to anhydrous salts.

ERRATUM.

Volume 32, No. 6, December, 1951. In the paper "The action of acridine compounds on cell division," by R. J. O'Connor, page 547, in Fig. 1 (page 549); *for* "aerobic glycolysis" *read* "respiration," and *vice versa*.

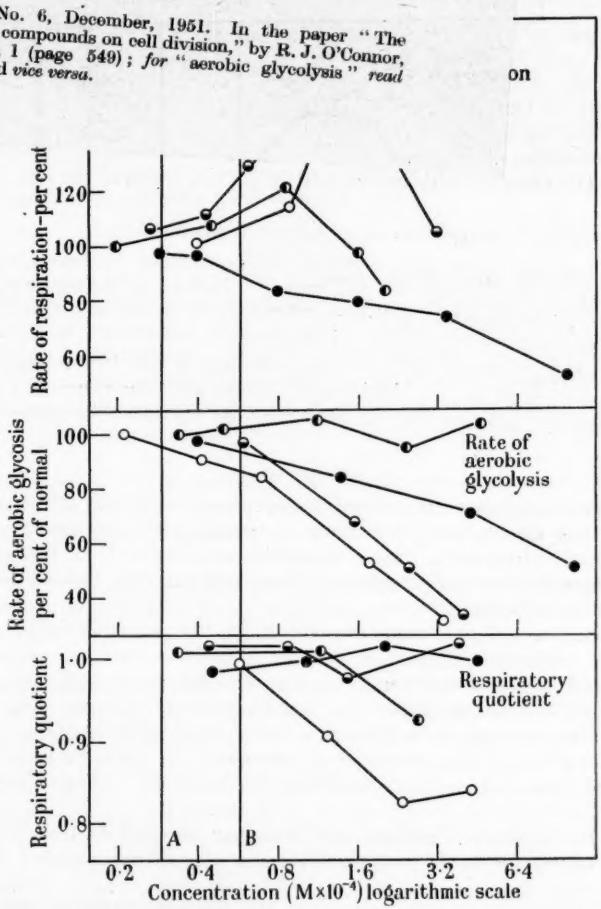

FIG. 1.—The effect of acridine compounds on the rates of respiration and aerobic glycolysis, and on respiratory quotient in the isolated midbrain of the 6-day chicken embryo. A–B indicates the range of effective mitotic concentrations (see text).

5-Aminoacridine hydrochloride.

5-Amino-2-trifluoromethylacridine hydrochloride.

5-Amino-1-phenyl-10-methylacridinium bromide.

5-*Cyclo*hexylaminoacridine hydrochloride.

TABLE I.—*The Effective Mitotic Conc*
the Isolated Midbrain

Compound.		
5-Aminoacridine hydrochloride	.	
5-Amino-2-trifluoromethylacridine hy		
5-Amino-1-phenyl-10-methylacridiniu... bromide	0·50 .	0·61
5-*Cyclo*hexylaminoacridine hydrochloride	0·40 .	0·33

Estimation of mitotic inhibition.

For each of the acridine compounds of Table I the concentration was determined which, in the isolated midbrain, caused fusion of the separated chromosomes in dividing cells. This change resembles that described by Dustin (1947) as pycnosis and has been described previously (O'Connor, 1949). To determine this "effective mitotic concentration" (E.M.C.) the technique previously described was used (O'Connor, 1950*b*). Portions of the isolated midbrain were exposed to graded concentrations of the acridine compounds for 90 min. at 37° C. For each acridine compound the E.M.C. was determined under two sets of conditions: firstly in a medium containing phosphate buffer in an atmosphere of oxygen; and secondly, in a medium containing bicarbonate in an atmosphere of 95 per cent oxygen plus 5 per cent carbon dioxide. These conditions corresponded to the conditions in which changes of respiration and aerobic glycolysis respectively were measured (see below).

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(*a*) For respiration and respiratory quotient: NaCl 0·90 g., KCl 0·02 g., $MgCl_2$ 0·02 g., $CaCl_2$ 0·02 g., glucose 0·10 g., water 100 ml. To this was added 10 c.c. of Sørensen's M/15 phosphate buffer to produce pH 7·4. The atmosphere in the divers was oxygen.

(*b*) For aerobic glycolysis: NaCl 0·90 g., KCl 0·02 g., $MgCl_2$ 0·02 g., $CaCl_2$ 0·02 g., $NaHCO_3$ 0·20 g., glucose 0·10 g., water 100 ml. The atmosphere in the divers was 95 per cent oxygen plus 5 per cent carbon dioxide. In both media the weights given refer to anhydrous salts.

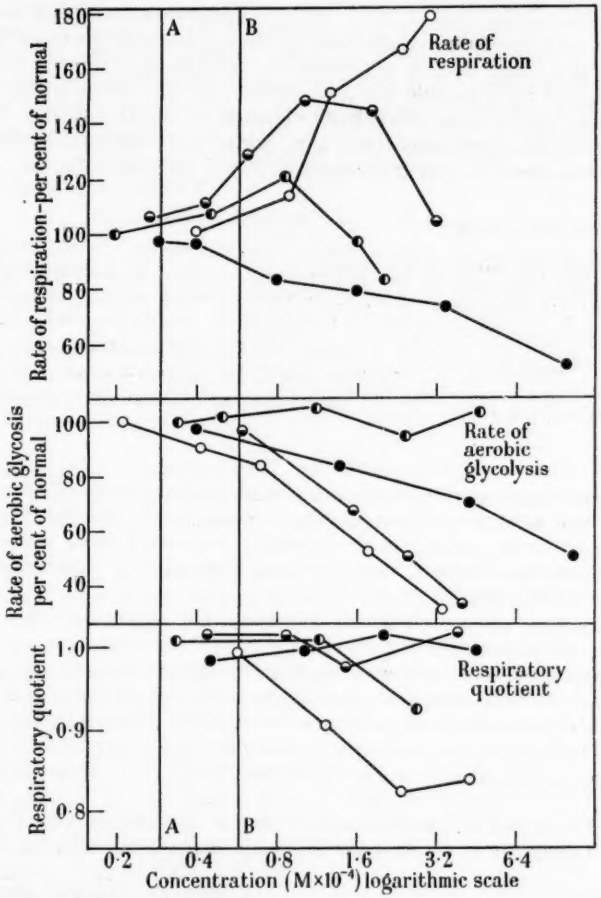

FIG. 1.—The effect of acridine compounds on the rates of respiration and aerobic glycolysis, and on respiratory quotient in the isolated midbrain of the 6-day chicken embryo. A–B indicates the range of effective mitotic concentrations (see text).

5-Aminoacridine hydrochloride.

5-Amino-2-trifluoromethylacridine hydrochloride.

5-Amino-1-phenyl-10-methylacridinium bromide.

5-*Cyclo*hexylaminoacridine hydrochloride.

RESULTS.

Effective mitotic concentration of the acridine compounds.

As described above, the E.M.C. for the acridine compounds was measured separately in each medium and the results obtained are set forth in Table I, each result being the mean of 4–6 experiments. In all experiments dilutions were made by steps of two so that any two results are not likely to be significantly different unless one is twice the other. Thus it is considered that the figures of Table I do not differ significantly and that, for all four compounds, in both media the range of the effective mitotic concentration is $0{\cdot}3$–$0{\cdot}6 \times 10^{-4}$ M.

The effect of acridine compounds on carbohydrate metabolism.

The effect of the same four acridine compounds on the rates of respiration and aerobic glycolysis and on the respiratory quotient is shown in Fig. 1. Each recorded point is the mean of 4–6 measurements, and on each figure the range of the effective mitotic concentration is indicated.

It is clear that with all four acridine compounds mitotic damage is produced in concentrations that do not significantly alter respiration, aerobic glycolysis or respiratory quotient, these having been used as criteria of carbohydrate metabolism.

DISCUSSION.

Although at higher concentrations the acridine compounds affect carbohydrate metabolism, the effects are variable in contrast with the relatively constant effect on the dividing cells. It is therefore concluded that the effect of the acridine compounds on the dividing cells is not directly due to alterations in carbohydrate metabolism.

The production of the pycnotic fusion of the separated chromosomes in dividing cells without inhibition of aerobic glycolysis contrasts with the effects of fluoride and iodoacetate, where similar changes were only produced when aerobic glycolysis was inhibited by approximately 50 per cent (O'Connor, 1950*c*). Similar investigations have now been made with urethane and nitrogen mustard, both of which also produced an association of pycnotic fusion of chromosomes and inhibition of aerobic glycolysis (see Table II). Thus there is a group

TABLE II.—*The Effect of Urethane and Nitrogen Mustard on Cell Division and Aerobic Glycolysis in the Isolated Midbrain of the 6-day Chicken Embryo.*

Compound.	E.M.C. in medium containing bicarbonate (M.).	Inhibition of aerobic glycolysis at E.M.C. (per cent).
Urethane	$8{\cdot}4 \times 10^{-2}$	40
Methyl bis(chlorethyl)-amine (nitrogen mustard)	$1{\cdot}7 \times 10^{-4}$	40

E.M.C. = effective mitotic concentration (see text).

of compounds which, in dividing cells, produce changes similar to those produced by acridine derivatives, but, in contrast with them, these changes are associated with inhibition of aerobic glycolysis.

In the normal development of the midbrain of the chicken embryo there is a close association between cell division and aerobic glycolysis (O'Connor, 1950*a*),

making it possible that energy necessary for cell division is derived from the latter. In this case it is likely that the energy is transmitted by the adenosine triphosphate mechanism, interference with which might produce changes in cell division similar to those produced by the inhibition of aerobic glycolysis. It is possible, therefore, that the effect of acridine compounds on cell division is due to interference with the adenosine triphosphate mechanism, and certain of the chemical properties of acridine compounds favour such a possibility. Wagner-Jauregg (1936) has demonstrated the formation of definite chemical compounds between acridine and the adenosine triphosphates, while McIlwain (1941) has shown a similar combination of euflavin with adenylic acid.

SUMMARY.

1. In the isolated midbrain of the chicken embryo four acridine compounds inhibit cell division without demonstrable effect on carbohydrate metabolism.

2. At concentrations higher than those necessary to inhibit cell division there are alterations in carbohydrate metabolism, as indicated by changes in the rates of aerobic glycolysis and respiration and in the respiratory quotient.

3. Equivalent concentrations of the compounds investigated vary considerably in their effects on carbohydrate metabolism, whereas their effects on cell division are similar.

4. The histological appearances of the dividing cells damaged by acridines are similar to those produced by certain other chemical substances which show inhibition of aerobic glycolysis at concentrations required to produce the changes in dividing cells.

5. The possibility is discussed that the acridine derivatives produce their effect on dividing cells by interfering with the adenosine triphosphate mechanism for energy transfer.

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ANTIBODY TO PURIFIED VIRUS INHIBITOR.

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Antibody to glyco-protein has been described from time to time, but the potency of the antigen is generally regarded as low (Goodner, 1925; Lewis and Wells, 1927) and the specificity of the antibody doubtful (Elliot, 1914), although, with improved methods of purification of biological materials, serological cross-reactions with heterologous mucins and mucoids and with other proteins of the homologous species have been gradually eliminated (Lewis and Wells, 1927; Hiscock, 1948). By taking advantage of the adjuvant method of Ramon, Richou and Staub (1937), and Freund, Casals-Ariet and Genghof (1940), it has been possible to prepare, without difficulty, immune sera to human urinary mucin, a substance estimated to be homogeneous by electrophoretic analysis, and known to be a suitable substrate for the enzymic action of influenza virus (Tamm and Horsfall, 1950). Work of a similar nature was begun by French and Gottschalk in this Institute in 1948 and mentioned in the Annual Report (1948–49), but was discontinued for lack of a pure antigen.

This paper describes the properties of the antibody thus obtained, which is noteworthy, firstly for its relative ease of preparation, secondly for its extreme specificity and, thirdly, for its unusual biological action.

MATERIALS AND METHODS.

Antigen.—1. For *in vitro* tests, urinary mucin, usually referred to as urinary inhibitor, was prepared by Mr. G. Ada, after the method of Tamm and Horsfall, and stored at 4° in a dilution of 6 to 12 mg. of urinary inhibitor per ml. of distilled water. From this stock suitable dilutions were made in saline.

2. For immunisation, a water-in-oil emulsion in liquid paraffin and sterile lanoline base was made from the antigen stock. The estimated content of the immunising agent was 2 to 4 mg. of purified inhibitor per ml. of emulsion.

Antibody.—The serum of immunised rabbits, bled ten days after the last injection of antigen, the serum being separated from the clot after 24 hours at 4°, inactivated by heating at 56° for thirty minutes, and stored at 4° with 1:10,000 merthiolate as a preservative.

Saline.—0·85 per cent sodium chloride in distilled water.

Serum-globulin.—Commercial preparations of human gamma-globulin.

Serum-albumin.—Prepared in this laboratory by solvent fractionation of human sera.

Influenza virus.—1. MEL: The Melbourne strain of influenza A (Burnet, 1935).

2. LEE: A strain of influenza B (Francis, 1940).

* In receipt of a grant from the Nuffield Foundation.

Both strains were grown in the allantoic cavity of 11-day-old chick embryos and were harvested fresh as required.

Indicator virus.—The above strains, suitably treated for conversion to the indicator state and referred to as T-MEL and H-LEE (Stone, 1949).

R.D.E.—Receptor-destroying enzyme, an extract of the 4Z strain of *Vibrio cholerae*, by the method of Burnet and Stone (1947).

Fowl cells.—Inhibitor-sensitive fowl cells (Anderson, 1948) collected from suitable adult fowls, washed thrice and made up to 1 per cent suspension in saline.

Precipitin tests.—The ring test was used (Medical Research Council, 1931) with antiserum in constant dilution in the lower layer, and falling dilutions of antigen in saline forming the superficial layer. Readings were made in two hours, after which no increase of titre was observed.

Inhibitor titration.—Falling dilutions of the sample of inhibitor were titrated against five agglutinating doses of indicator virus by the method of Burnet (1948).

EXPERIMENTAL.

The course of immunisation.

Two rabbits, approximately twelve months old, were chosen, whose serum did not produce a precipitate with the antigen in any dilution. Three subcutaneous injections of antigen emulsion were given at intervals of ten days, the first two of 1 ml. each, the third of two separately spaced quantities of 1 ml. each. The total dose of antigen was thus between 12 and 24 mg. Test bleedings were made after each inoculation and 40 ml. blood removed from an ear vein, when the precipitin test showed a reasonably high titre of antibody. The serum was treated and stored as has been described.

The specificity of the antibody.

Evidence of the specificity of the antibody was first sought and the results of precipitin tests appear in Table I. Serum dilution 1:5 gave highest titres with specific antigen, and this dilution was adopted for all tests except those likely to involve small quantities of antigen, e.g., in testing non-concentrated urine, when a dilution of 1:1 in saline was preferred. Purified preparations of antigens were tested over a range of 1:10 to 1:10,240, crude preparations from 1:2 to 1:1280.

TABLE I.—*The Specificity of the Antibody : Precipitin Test.*

Antiserum.	Dilution of antiserum.	Titre against urine inhibitor : antigen dilution.	Negative, at dilutions 1:10 to 1:10,240, with—	
			Homologous.	Heterologous.
Serum 1 : Pre-inoculation	1:1 and 1:5	*nil*	Gamma globulin	Bovine salivary mucin
Serum 1 : 30th day	1:1 and 1:5	1:5120	Serum albumin	Ovomucin
Serum 2 : Pre-inoculation	1:1 and 1:5	*nil*	Whole serum	Urinary mucin of sheep, horse, and mouse
Serum 2 : 30th day	1:1 and 1:2	1:640	Salivary mucin	—
	1:5	1:1280	Ovarian cyst mucin	—

It will be seen that the antibody is quite specific for purified urinary inhibitor, no cross-reaction being detected either with human proteins or with mucoproteins of other species.

Antigen-antibody reaction with degraded antigen.

Since the inhibitor action of urinary mucin on virus haemagglutination is destroyed by active virus and by receptor-destroying enzyme, a comparison was made between the behaviour of specific inhibitor in the precipitin test before treatment with these reagents and its behaviour after treatment. The method consists briefly in digesting at 37° equal quantities of urinary inhibitor with equal amounts of each virus preparation and with an equivalent quantity of RDE, until all inhibitor activity is destroyed. The inhibitor activity is tested with indicator virus in the usual way after inactivating the virus—or enzyme—inhibitor digest by heating at 65° for thirty minutes, a procedure which destroys enzymic and haemagglutinating activity. The treated inhibitor, now without inhibitory activity, is put up in doubling dilutions against specific antiserum. A specimen incubated with an equal quantity of inactivated RDE and subjected to the same procedure serves as a control.

TABLE II.—*Degraded Urinary Inhibitor as Antigen : Precipitin Test.*

	Antigen treated with—	Inhibitor titre of antigen.	Antiserum.	Antigen dilutions. 40.	80.	160.	320.
1.	80 AD's MEL	*nil*	1	+	++	++	tr.
			2	+	±±	+	−
2.	80 AD's Lee	,,	1	+	++	+	tr.
			2	+	+	+	−
3.	100 units RDE	,,	1	+	+	+	±
			2	+	+	±	tr.
4.	100 units RDE inactivated	1:1280	1	+	+	+	±
			2	+	+	±	tr.

tr., ± +, ±±, ++ = increasing degree of density of precipitin ring.

As is seen in Table II, there is no apparent decrease in the efficacy as precipitinogen of virus-treated or enzyme-treated urinary inhibitor. In fact, during the experiment, precipitin rings with treated inhibitor appeared sooner, and were more sharply defined, than those of the untreated mucoprotein, but the titres were never higher.

The effect of antigen-antibody union on the inhibitory action of urinary mucin.

Union of urinary inhibitor with specific antibody neutralises the haemagglutinin inhibiting property of the antigen. This applies whether there is a visible precipitate of antigen-antibody complex or not. The effect is best shown as an experimental titration, the rationale of which requires some explanation.

The potency of urinary inhibitor may be estimated by titration against a fixed dose of indicator virus, five agglutinating doses (5 AD's), according to the method quoted above (Burnet, 1948). That amount of inhibitor which will just inhibit the agglutinating action of 5 AD's of indicator virus may easily be prepared and is referred to as one inhibiting dose (1 ID) of inhibitor. If now, to a series of tubes each containing 2 ID's of inhibitor, 5 AD's of indicator virus are added, and, after thirty minutes, one volume of a 1 per cent suspension of sensitive fowl cells, no haemagglutination will take place. But if to each tube is added an equal volume of falling dilutions of antibody and, after suitable intervals, 5 AD's of indicator virus and one volume of 1 per cent fowl cells, haemagglutination will take place whenever antibody combines with, and so neutralises, inhibitor. At a

point where antibody is too dilute to counteract the union of virus and urinary inhibitor, inhibition of haemagglutination reappears. This may be taken as the end-point of the titration of antiserum. The titration may be referred to as an anti-inhibitor titration. There is one complication. The specific antibody, like all sera, contains non-specific virus-inhibitory substances (Francis inhibitor) which may exceed the titre of specific immune body and so, by inhibiting haemagglutination throughout, mask its anti-inhibitor action. This effect may be eliminated by the pre-treatment of the serum with RDE and subsequent inactivation of the enzyme at 65° for thirty minutes before carrying out the anti-inhibitor test proper.

In order to illustrate all the preceding points, the succeeding table shows experimental readings in full (Table III).

TABLE III.—*The Anti-inhibitor Action of the Immune Serum.*

Serum.	Dilution of antiserum (v. 2 ID's of antigen).												
	2.	4.	8.	16.	32.	64.	128.	256.	512	1024.	2048.	4096.	8192.
Pre-inoculation 1 .	−	−	−	−	−	−	−	−	−	−	−	−	−
„ 2 .	−	−	−	−	−	−	−	−	−	−	−	−	−
Immune 1 . . .	−	−	−	−	−	−	−	tr.	++	++	++	+	−
„ 2 . . .	−	−	−	−	−	−	tr.	+⁺	++	++	++	tr.	−
Immune 1 + RDE .	−	−	+	++	++	++	++	++	++	++	++	−	−
„ 2 „ .	−	−	±±	++	++	++	++	++	++	++	−	−	−
	Non-specific inhibitory titre of antiserum.												
Immune 1 . . .	−	−	−	−	−	−	±	++	++	..	..	..	..
„ 2 . . .	−	−	−	−	−	tr.	++	++	++	..	..	..	..
Immune 1 + RDE .	−	−	tr.	++	++	++	++	++	++	..	..	..	..
„ 2 „ .	−	±	++	++	++	++	++	++	++	..	..	..	..

−, ±, +, +⁺, ±±, ++ = ascending degrees of agglutination. + RDE = RDE-treated.

Pre-inoculation sera have no action on 2 ID's of inhibitor. Inhibition of haemagglutination is, therefore, complete. Immune sera neutralise the biological action of urinary inhibitor, but, owing to the presence of non-specific inhibitors in the sera, this effect, as shown by the appearance of haemagglutination, is only visible above titres of 1:128. RDE-treated immune sera, on the other hand, reveal the anti-inhibitor effect from titres of 1:8 upwards.

A serum with an anti-inhibitor titre of 2000 against 2 ID's of urinary inhibitor was found to be quite negative against 2 ID's of ovarian cyst mucin, human serum, human salivary mucin and ovomucin. Thus the specificity of the anti-inhibitor test corresponds to that of the precipitin test.

Anti-inhibitor titrations with degraded urinary inhibitor.

Precipitin tests indicated that the combining power of urinary mucoprotein with antibody was unaffected by treatment of the antigen with RDE. This finding is fully confirmed by using the anti-inhibitor titration to estimate the amount of antibody absorbed by enzyme-treated and by untreated urinary inhibitor. The steps of the experiment are outlined below :

1. To 0·5 ml. antiserum add 0·5 ml. of enzyme-treated urinary inhibitor prepared from 4000 ID's of pure inhibitor. To a second 0·5 ml. antiserum add 0·5 ml., 4000 ID's, of inhibitor which has, as a necessary control, been incubated with non-active RDE and subjected to the same heat treatment as the first specimen.

2. Incubate the mixtures 30 min. at 37° and remove the antigen-antibody precipitate by centrifuging at 4000 r.p.m. for 5 min.

3. Remove the supernatant absorbed sera, treat each with RDE in order to destroy non-specific inhibitor and any excess antigen. Inactivate the RDE for 30 min. at 65°.

4. Titrate the anti-inhibitor value of each specimen.

5. Titrate the non-specific inhibitor value of each specimen.

The results of such an experiment appear in Table IV*a*. It is apparent that enzyme-treated urinary inhibitor combines readily with specific antibody and absorbs almost as much from the immune serum as does non-treated urinary inhibitor. In other words, destruction of virus-enzyme substrate fails to modify the serological specificity of the urinary inhibitor.

TABLE IV*a*.—*The Neutralisation of Anti-inhibitor by Intact and Degraded Specific Antigen.*

Serum.	Absorbed with inhibitor.	Treated by—	Inhibitory titre.	Anti-inhibitor titre (v. 2 ID's inhibitor). 8.	16.	32.	64.	128.	256.	512.	1024.	2048.
1	4000 ID's	RDE and heat 56° for 30 min.	*nil*	++	++	−	−	−	−	−	−	−
	4000 ID's + RDE		,,	++	++	+⁺	tr.	−	−	−	−	−
	Saline		,,	+⁺	±±	++	++	++	++	++	++	±
2	4000 ID's		,,	−	−	−	−	−	−	−	−	−
	4000 ID's + RDE		,,	−	−	−	−	−	−	−	−	−
	Saline		,,	+⁺	++	++	++	++	++	++	tr.	−

TABLE IV*b*.—*The Simultaneous Absorption of Anti-inhibitor and Precipitin by Specific Antigen.*

Serum.	Absorbed with—	Precipitin titre.	Anti-inhibitor titre.
1	2000 ID's	40	1400
	,, + RDE*	40	1400
	4000 ID's	*nil*	*nil*
	,, + RDE	,,	12
	Saline	160	4000

* + RDE = treated with RDE.

Some correlation between the absorption of precipitin and anti-inhibitor values is shown in Table IV*b*.

DISCUSSION.

In addition to the immunological interest of the preparation of antibody to a truly homogeneous mucoprotein, which has been mentioned in the introduction to this paper, the antigen-antibody reaction described permits some deductions to be made about the nature of urinary inhibitor and the relationship between virus-enzyme substrate groupings in the molecule and antigenic configuration.

Purified urinary mucin is antigenically distinct from human serum proteins, from ovarian cyst mucin and from salivary mucin. It is also unrelated sero-

logically to mucoproteins of certain mammalian tissues, and also to certain mammalian urinary mucins which are known to be suitable substrates for virus enzymic action.

The experimental findings show that the structural configuration responsible for antigenicity is not destroyed when the virus-enzyme substrate is removed by receptor-destroying enzyme, yet antigen-antibody union neutralizes the inhibitor action of the preparation. These facts are supported by the primary observation that no cross-reactions occur between antibody to human urinary mucin and other mucoproteins which do contain virus-enzyme substrate. Also, although it appears that the enzyme-treated inhibitor will in fact absorb slightly less antibody than urinary inhibitor which has not been so degraded, this is a quantitative alteration in the sense that an excess of treated inhibitor always removes all trace of anti-inhibitor. That is to say, there is no separable fraction of antibody whose origin is attributable to the virus-enzyme substrate component of urinary inhibitor.

It is clear, then, that the preparation of urinary inhibitor used as antigen contains a virus-inhibitor substrate which is non-antigenic and which may be related to the mucoprotein in one of several ways.

Firstly, the inhibitor-substrate may be a substance distinct from the mucoprotein, becoming actively absorbed in the antigen-antibody complex.

Secondly, the non-antigenic inhibitor-substrate may be firmly absorbed to the antigen itself.

Thirdly, inhibition of virus-haemagglutination may be a function of the antigenic macro-molecule dependent on molecular groupings which are not antigenic determinants.

A decision between these possibilities will depend essentially on further physico-chemical studies of the material. If it can be established that there is but one molecular species in all the preparations, the third possibility offers the best possible explanation of the facts presented in this paper. For example, the indication, from results of absorption of anti-inhibitor, that degraded antigen is qualitatively identical with, but quantitatively slightly less active than, intact antigen, suggests that the inhibitor macro-molecule is not quite unaltered by the action of virus or enzyme and is in favour of the substrate being an integral part of the molecule. It is analogous to the findings of Burnet and Anderson (1947) that species-specific antigens on certain erythrocytes are quantitatively but not qualitatively altered by treatment with RDE, and to the observations that blood-group A, B and O specificity of human red cells and the specificity of highly purified blood-group A substance are unaltered by the action of RDE (Burnet, McCrea and Anderson, 1947), although such treatment removes influenza virus receptors from the former and destroys the powerful virus-inhibitory action of the latter.

SUMMARY.

1. The preparation of specific antibody to a homogeneous mucoprotein, human urinary virus-inhibitor, is described.

2. Its unusual biological action in neutralizing the inhibition of virus-haemagglutination is established.

3. The antigenicity of the mucoprotein is independent of the presence of virus-enzyme substrate groupings which are probably part of the inhibitor macro-molecule.

The author wishes to express his gratitude to Sir Macfarlane Burnet, F.R.S., who suggested and guided this work, and to Mr. G. L. Ada and members of his department, who supplied many samples of purified muco-protein.

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A HEPATITIS VIRUS OF MICE.

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DURING the autumn of 1950 considerable losses were experienced in the breeding stock of a strain of white mice (Parkes (P) strain) maintained at the National Institute for Medical Research. Owing to structural repairs to the animal house, the P mice had been confined to half the space normally allotted to them, and central heating was temporarily unavailable. The majority of dead mice had massive caseous lesions of the liver, a substantial minority had the liver lesions characteristic of infection with *Bacillus piliformis* and a few had indefinite lesions. From the caseous liver lesions were isolated Gram-positive organisms, possibly enterococci. While intraperitoneal injection of mice with cultures of these organisms occasionally reproduced the caseous liver lesions, feeding cultures regularly failed to do so. It therefore seemed possible that the primary causal agent might be a virus which damaged the liver sufficiently to render it susceptible to a variety of secondary invaders. Accordingly, membrane filtrates of liver extract from dead P mice were passed as described below in young weaned mice of another (VS) strain maintained at the Institute, since it was felt that the P mice might have become immune to any agent originally responsible for the outbreak. This VS strain of mice originated from the Rockefeller albino mice selectively bred by Webster (1937) for resistance to bacteria and susceptibility to viruses. By the fourth pass, liver lesions were quite evident at one week in killed VS mice and by the fifth pass there were deaths. The infective agent will be termed mouse hepatitis virus (MHV).

The purpose of this communication is to give some account of the disease produced in mice by MHV and to describe its general properties.

TECHNIQUE.

Extracts of liver and kidneys were prepared from sick or moribund mice infected 5 or 6 days previously by mincing the organs and grinding with sterile powdered glass or quartz with pestle and mortar. The tissue emulsion was suspended in 5 or 10 per cent horse serum broth—5 ml. for the pooled liver and kidneys of one mouse. After sedimentation by gravity or light centrifugation to remove the coarser particles, the supernatant was ready for use. All dilutions were made in serum broth, the dose per mouse being from 0·15 ml. to 0·30 ml. of ten-fold dilutions of the extract.

For filtration, the crude extract was centrifuged for 10 min. at 3000 r.p.m. and filtered by positive pressure through a 2·0 μ A.P.D. (average pore diameter) gradocol membrane, followed by filtration through a 1·0μ upon a 0·7μ membrane. Such filtrates proved bacteriologically sterile, nor was it possible to grow from them organisms of the pleuro-pneumonia group.

Newly weaned VS mice of 8–11 g. (aged 3–4 weeks) infected by intraperitonea inoculation proved to be the most convenient and sensitive indicator for the presence of MHV and were generally used for this purpose.

THE DISEASE IN MICE.

The symptoms in affected mice.—Infected VS mice (8–11 g.) appear slightly unwell after an incubation period varying from 4 to 7 days according to the magnitude of the infecting dose. Thereafter, the mice rapidly deteriorate, becoming very ill, often with muscular tremors; if twisted by the tail they may go into a tonic spasm and die. The urine is markedly yellow, red or brown, staining the coat in the perineal region. Mice becoming very ill die within a few hours, but slightly ill mice may recover. The mortality in VS mice of less than 14 g. weight approaches 100 per cent, the highest death-rate occurring on the fifth day after infection with a heavy dose, on the seventh or eighth day with a dose which is only just lethal.

The macroscopic lesions in mice.—On post-mortem examination the most striking abnormality is found in the liver. This is swollen and friable; it may be pale yellow and mottled with petechial haemorrhages, or it may be brown and similarly mottled. Its appearances do, in fact, simulate those associated with ectromelia. The spleen is often somewhat swollen and dark in colour. The kidneys may be swollen and pale, especially in the more chronic cases. Susceptible mice infected with a small dose and showing no definite symptoms of illness frequently manifest striking liver lesions if killed between the seventh and tenth day after infection. In mice which appear to have recovered the liver presents an irregular surface as if there were uneven shrinkage of fibrous tissue.

Histological changes.—Only preliminary observations on histology have yet been made. The earliest liver lesions have been found 3 days after intraperitoneal inoculation; they take the form of small round cytoplasmic inclusion bodies in cells of liver parenchyma; they are pink in tissue stained with haematoxylin and eosin. Study of sections obtained on subsequent days suggests that the inclusions rapidly enlarge, and that the cytoplasm is soon filled by deeply eosinophilic material. Since necrotic liver cells may in any case become eosinophilic, it is hard to say how far all the deeply pink-staining material is of the same nature as the inclusions observed earlier. At the same time, degenerative nuclear changes occur, first margination of chromatin and then nuclear fragmentation. The affected cells appear to run together, forming deep pink masses. These focal necroses are scattered through the liver substance; they may or may not be in close relationship to blood vessels. Infrequently, intranuclear inclusions have been found in numbers; they may be due, not to the same agent, but to that described by Findlay (1932) and by Pavilanis and Lépine (1949). In mice which die the liver necrosis extends until little recognisable structure remains. In those which recover a cellular reaction rapidly sets in. Polymorphs and mononuclear cells appear in the foci; the masses of pink material shrink and are walled off by giant cells; proliferation of bile ducts is very active and mitoses of hepatic cells are numerous. After 15 days livers of recovered mice may show a diffuse proliferation of young connective tissue, largely intercellular, but showing at times a coarser architecture.

In some mice pink cytoplasmic inclusions, together with some epithelial necrosis, have been found in kidney tubules. Gross kidney damage has been

rare, though occasionally very extensive in older P mice. Focal meningeal lesions have been seen, but systematic histological study has still to be carried out.

Properties of the Virus.

The LD_{50} and size of MHV.—The LD_{50} of liver-kidney extract was determined by the method of Reed and Muench (1938) from the mortality amongst groups of VS mice (8–11 g.) inoculated intraperitoneally with ten-fold dilutions from 10^0 to 10^{-6}; it was within the range of 10^{-4} to 10^{-5} ml. both for unfiltered extracts and for filtrates through membranes of A.P.D. 0·7–0·8μ. Using such filtrates for filtration through finer membranes, we found that the virus would pass through 0·35μ and 0·3μ membranes, but failed to pass through membranes of 0·25μ. The protocols of these experiments are given in Table I. These results indicate that MHV is one of the larger viruses, falling in the range 130–180 mμ; it is, therefore, of about the same size as vaccinia. It may also be observed in Table I that the average survival time increases somewhat regularly with increasing dilution of virus.

TABLE I.—*The Filterability of MHV.*

*APD of membrane filter.	Dose injected i/p (ml.).	Numbers dying.	Average survival time of mice which died (days).
0·75μ	0·25 × 10^{-6}	0/5	–
,,	0·25 × 10^{-5}	1/6	8
,,	0·25 × 10^{-4}	1/6	8
,,	0·25 × 10^{-3}	3/6	7·3
,,	0·25 × 10^{-2}	6/6	6·7
,,	0·25 × 10^{-1}	5/5	6·2
,,	0·25 × 10^{0}	5/5 (6/6)	5·6 (5·0)
0·35μ	0·3 × 10^{0}	5/5 (6/6)	7·0 (6·0)
0·30μ	0·3 × 10^{0}	6/6	7·2
0·25μ	0·3 × 10^{0}	0/5 (0/6)	– (–)

The LD_{50} calculated from this experiment is $1·3 \times 10^{-4}$ ml.
The bracketed figures refer to a second filtration experiment.
* APD = average pore diameter of gradocol membrane filters.

The infectivity of blood, urine and faeces.—Blood drawn into heparin from the hearts of anaesthetized mice moribund of the disease proved infective, having an LD_{50} certainly less than 1 ml. × 10^{-2} in one experiment and 2 ml. × 10^{-3} in another. Urine obtained direct from the bladders of killed moribund mice infected a proportion of mice in a dose of 5 ml. × 10^{-3} in one experiment. In three other experiments a dose of 3 ml. × 10^{-2} failed to infect any of 6 mice. A 0·7μ membrane filtrate of extract prepared from faeces taken from the rectum of killed moribund mice proved infective for susceptible VS mice in two of three experiments. It thus seems that the blood of infected mice contains a relatively high concentration of virus, and that virus may be present in the faeces and occasionally in the urine.

Other routes of infecting mice.—Since nervous symptoms are sometimes shown by sick mice, we studied the effect of brain-to-brain passage of the virus in young VS mice to discover whether its neurotropism could be easily enhanced. It was found that, although the virus was maintained for four brain passes, the character of the disease produced did not vary. The lengthened time of survival (8–9 days) and the fact that some mice survived suggested that brain extract may have been infective on account of the blood contained in it. Young VS mice were also successfully infected by subcutaneous and intranasal inoculation. One attempt

has been made to infect mice by mixing about 10^4 LD_{50} of MHV with the diet fed to 12 young VS mice on 3 consecutive days. Two mice died with typical lesions 12 and 13 days after the first feeding with virus. The disease did not pass from mouse to mouse by cage contact as a lethal infection. We are now studying the question of whether it does so as an inapparent immunizing infection.

Attempts to infect other laboratory animals.—Attempts were made to infect cotton-rats, guinea-pigs and hamsters by intraperitoneal inoculation of a maximum amount of active extract; no symptoms or lesions were produced in these species. Liver and kidney extract from two young rabbits infected with active virus was passed to susceptible mice and to further rabbits without apparent result. In rats, two litters of 2–4 days were infected by intraperitoneal injection, and liver-kidney extract from them was passed 7 days later to susceptible mice and two more litters of rats; no symptoms were observed within a period of a week after infection, at which time no lesions were observed in the killed mice or rats. The adaptability of the virus to rats is, however, still under investigation. Unsuccessful attempts were also made to infect cats, ferrets and a dog.

Attempts to grow the virus in fertile eggs and tissue cultures.—Unsuccessful attempts were made to infect fertile eggs by inoculation on to the chorio-allantoic membrane, into the yolk sac and into the allantoic cavity. Failure to produce symptoms or lesions in susceptible mice inoculated with material harvested from the eggs was taken as indicating absence of growth of the virus in the eggs. Inoculations were also made into the amnion of fertile eggs; lesions were not produced in mice by the amniotic fluid harvested from them except minimal lesions at the first pass; there was possibly some survival of the original inoculated virus. No growth or survival of virus has been obtained in tissue cultures, using chick and mouse embryos in Carrel flasks and roller tubes, by ourselves or by our colleague, Dr. T. H. Flewett.

Resistance of MHV.—The relative absence of resistance of the virus to heat, ether, 50 per cent glycerol and storage at $-65°$, as detailed in Table II, seem to distinguish it from human infectious hepatitis. Results of storage at $-65°$ have, however, been more erratic than the table indicates. In a preliminary experiment freeze-dried liver mince and liver extract were practically inactive after 9 days' storage at 2°. The preservation of the virus by freeze-drying is, however, still under investigation.

Neutralization of MHV.—Experiments were conducted to see whether MHV was neutralized by the sera from recovered and hyperimmunized mice. Normal sera were obtained from groups of 12–20 mice of 18–20 g., bled out under chloroform anaesthesia. Two such batches were prepared in this way from VS mice and from P mice.

Hyperimmunized mice were prepared from survivors of groups of VS mice of 10–12 g. infected by intraperitoneal inoculation of virus, reinfected 7–14 days later with 0·5 ml. of undiluted, unfiltered, active liver-kidney extract and bled out 14–20 days later. Two such batches were prepared, the mice being bled out 14 days (S_1) and 20 days (S_2) after the last injection. S_3 was prepared by bleeding out the 6 survivors of a group of 12 (14–16 g.) young adult VS mice infected with 10^3 LD_{50} 14 days before. S_4 was prepared by bleeding out the 13 survivors of a group of 15 fully grown VS mice infected with a similar dose 14 days before.

TABLE II.—*Stability of MHV.*

Treatment.	LD_{50} of untreated control extract.	LD_{50} of treated portion of same extract.	Remarks.
Heated to 56° for 30 min.:			
(1)	$<0{\cdot}25$ ml. $\times 10^{-2}$	$>0{\cdot}25$ ml.	Inactivated; no symptoms or lesions produced in mice by 0·25 ml.
(2)	$0{\cdot}25$ ml. $\times 10^{-2}$	$>0{\cdot}25$ ml.	
Kept in contact with 20 per cent ether overnight at +2°	$<0{\cdot}25$ ml. $\times 10^{-3}$	$>0{\cdot}25$ ml.	Inactivated; 5/6 mice receiving 0·25 ml. had no symptoms or lesions. One died with inconclusive lesions.
Liver suspended in 50 per cent glycerol for 6 weeks at + 2°	$<0{\cdot}25$ ml. $\times 10^{-2}$	$>0{\cdot}25$ ml.	Practically inactivated; 3/5 mice receiving 0·25 ml. had liver lesions from which one had died.
Stored at −65°;			
(*a*) For 9 days	$1{\cdot}3$ ml. $\times 10^{-4}$	1 ml. $\times 10^{-4}$	Titre unchanged.
(*b*) For 2 months	$1{\cdot}3$ ml. $\times 10^{-4}$	3 ml. $\times 10^{-3}$	Titre 1/23 original titre.

TABLE III.—*Neutralization of MHV by Normal and Immune Sera.*

Serum.	*Death rates of mice infected with virus-serum mixtures containing LD_{50}s of virus as hereunder:			
	10^0–10^1	10^1–10^2	10^2–10^3	10^3–10^4
Normal VS serum	3/5	5/5	n.t.	n.t.
Normal P serum	3/5	5/5	n.t.	n.t.
Immune VS serum (S_1)	0/6	0/6	n.t.	5/5
„ „ (S_2)	1/5	5/5	n.t.	n.t.
„ „ (S_3)	0/4	4/5	5/5	5/5
„ „ (S_4)	0/5	1/5	3/4	5/5

Numerators represent mice which died, denominators total mice in group.
n.t. = not tested.
The dose of serum was 0.1 ml. per mouse mixed beforehand with the appropriate doses of virus.
* Control mice were inoculated with dilutions of virus without mouse serum and from the results with these the number of LD_{50}s of virus in the mixtures was calculated.

The method of carrying out the neutralization test was to mix 0·1 ml. of unheated normal or immune serum with 0·15 ml. of ten-fold dilutions of active extract and, after letting stand ½–1½ hrs. at 2°, to inject each dilution intraperitoneally into groups of 5 or 6 susceptible VS mice (8–12 g. weight). The LD_{50} of the active extract had to be determined at the same time owing to the lability of the virus.

It is clear from Table III that neutralizing antibody cannot be detected in the sera from normal VS or normal P mice by the method adopted. The sera, S_2 and S_3, from recovered and hyperimmunized VS mice show no significant amount of detectable antibody; S_1 and S_4 do show detectable antibody, although the amount is small. It seems, therefore, that the antibody response to MHV is slight, and this is in harmony with the fact that virus has been recovered from the blood of mature carrier P mice (see below).

Age and Strain Susceptibility in Mice.

It was soon noticed that the susceptibility of VS mice became much less after they had attained 14 g. in weight, that is, after about 4–5 weeks of age; also that the majority of newly weaned mice of the P strain did not succumb

to inoculation with a heavy dose. These findings were confirmed and extended to three other strains of mice, the C3H, the C57 (kindly supplied by Dr. J. Craigie), and a stock of Swiss mice, separate from the VS mice, which are maintained at the National Institute for Medical Research. These results are shown in Table IV.

TABLE IV.—*The Relationship of Mortality to Age and Strain of Mice.*

Strain of mice.	Mortality in mice aged—		
	2–4 days.	3–4 wks. (9–14 g.).	6 wks. or more (>16 g.)
VS	36/36 (100%)	186/193 (96%)	4/25 (16%)
P	38/57 (67%)	21/78 (27%)	3/20 (15%)
C_3H	6/6	4/15	n.t.
C_{57}	5/5	4/15	n.t.
Swiss	n.t.	3/12	n.t.

n.t. = not tested.
Inoculated intraperitoneally with 0·1 to 0·02 ml. active liver-kidney extract.

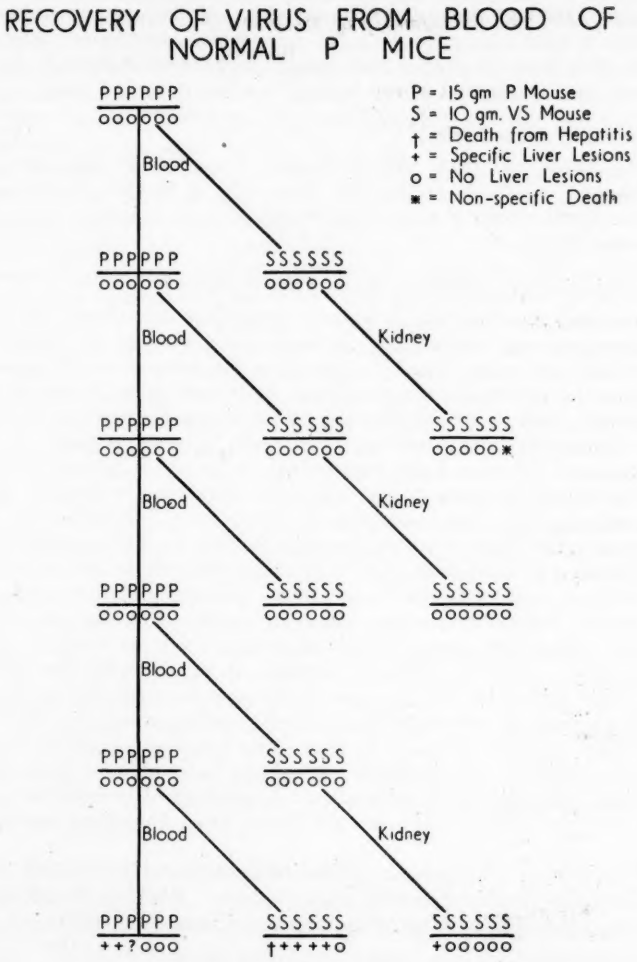
It is apparent that VS mice were uniformly susceptible until they attained a weight of 14 g. Between 14–16 g. the mortality was 6/12 (a result not appearing in the table), and above 16 g. more than 80 per cent survived challenge. For P mice, while the resistance was least shortly after birth and increased with age, mice of all ages showed some resistance. The mortality in P mice of 3–4 weeks old has since shown some tendency to rise. In experiments performed between February and May, 1951, the mortality was 0/10, 1/6, 2/6, 0/6 and 1/15; while in experiments performed in late June and July, it was 13/30 and 4/5 respectively. Throughout the period there appeared to be no change in the virus as judged by results in young VS mice. The resistance of the other strains would seem to resemble that of P mice, although the data are scantier for them. At any rate, it seems that of the strains tested, only VS mice remain highly susceptible at weaning time and for the first week or so afterwards.

Activation of Latent Virus.

In the course of an endeavour to discover whence the mouse hepatitis virus came, the following experiments were carried out: Three normal P mice weighing about 15 g. were killed and their kidneys and livers ground separately in 10 per cent horse-serum broth to make approximately 10 per cent suspensions. Kidney suspension in 0·25 ml. quantity was inoculated intraperitoneally into 6 more P mice, and liver suspension similarly to 6 others. The mice were killed after 7 days and the procedure repeated. Two series were kept going, liver suspensions being used in one, kidney suspensions in the other. Three serial passages were thus carried out through P mice, but at the fourth pass VS mice were used instead. Eight days later 4/6 mice in the kidney series were dead with typical hepatitis, confirmed histologically, and a fifth had extensive liver lesions. Passage to further VS mice was successful. In the liver series 2/4 mice were dead with typical lesions after 8 days.

This experiment left it uncertain whether several serial passes through P mice were necessary, or whether at any stage passage to VS mice would have revealed virus activity. Accordingly, passages were made through P mice as before, with use of kidney suspensions only, and at each pass inoculation was made also into VS mice. In this experiment all mice, whether P or VS, remained negative until, as happened before, deaths from hepatitis occurred in VS mice after 4

passages. In a third trial, younger (10 g.) VS mice were used and only 3 passages were necessary to produce specific deaths; on this occasion all mice died at the third passage, both the 10 g. VS and the 15 g. P. One of the latter showed extensive necrosis of voluntary muscles, a picture not encountered otherwise.



1. P passage with blood or kidney suspension as shown, at 7 day intervals.

FIGURE.

Next, kidney suspensions of normal P mice were injected intraperitoneally into 10 g. VS mice and serial passage carried on as usual but in young VS mice only. Hepatitis turned up after 3 passes.

Virus in blood of normal mice.

The possibility existed that passage from any organ might succeed because of presence of virus in blood of carrier mice. Heparinized heart blood of 6 normal P mice was therefore given intraperitoneally to 6 more P mice. These were bled after 7 days and passage thus carried on serially. No lesions appeared in the first 4 serial lots of P mice nor in the 10 g. VS mice which were subinoculated at each stage. But after 5 passes deaths from hepatitis occurred in both P and VS mice. Further details are contained in the figure.

Virus in faeces of normal mice.

Virus was recovered from filtered faeces of normal P mice after only one "blind passage" through young VS mice. In a further experiment faeces filtrates from 6 individual P mice were tested, but no infection was apparent in second-passage mice.

Do carriers exist in mice other than those of the P strain?

When starting material was a kidney suspension of normal 15 g. VS mice, 6 serial passages were carried out in such mice without revealing virus. A repetition of this test using 10 g. VS mice gave the same result (6 passes made at 5-day intervals). Similarly, no virus was activated after 4 passes when C_3H or C_{57} mice were used to furnish starting material and for further passage.

Though deliberate cross-infection experiments have as yet given no conclusive results, occasional VS mice have succumbed to hepatitis under conditions suggesting a disease of different origin. We are therefore cautious in interpreting the above experiments, but inasmuch as "activation" experiments were conducted in a room free from other sources of infection, we feel justified in accepting most of the results at their face value. We thus tentatively conclude that mouse hepatitis virus is carried in the blood, liver, kidneys and faeces of apparently normal P mice; conceivably some organs are active only because of the blood they contain. The high susceptibility of young VS mice enables us to detect this carrier-state. Several passages through P or VS mice are necessary to reveal it. This might be (in the case of P mice) because the chances increase with every passage of encountering a carrier which would furnish virus; or because P mice resemble VS mice in that serial passage permits a trace of virus to increase in quantity or virulence. We have been able to show that mouse hepatitis virus multiplies in P mice after intraperitoneal inoculation, even when no lesions become evident; the second factor must therefore certainly play a part.

Recent experiments indicate that after intraperitoneal inoculation into P mice, virus is maximal in the kidneys after about 5 days. We may therefore have been waiting beyond the optimal time in making serial passes at weekly intervals.

Some Other Aspects of MHV.

Relationship of MHV to other viruses.—Of the viruses characteristically producing hepatitis in man and animals, MHV differs in its larger size, greater lability and in other ways from the viruses of yellow fever and rift valley fever. It differs from the viruses of infectious and homologous serum hepatitis of man in its short incubation period, its lability—notably its thermolability and its lack of resistance to ether, drying and storage at $-60°$. Fox encephalitis (Rubarth's

canine hepatitis) is also distinguished from MHV by the regular presence of intranuclear inclusion bodies and by different host specificities. There remain a number of reports of viral hepatitis in mice, either spontaneous or believed to have been produced with infectious material of human origin. Apart from the condition already mentioned characterized by an unusual type of intranuclear inclusion bodies (Findlay, 1932, and Pavilanis and Lépine, 1949), Nicolau and Ruge (1944) have described an agent believed to have derived from human infective jaundice and producing histological changes in mice. These changes do not resemble those produced by MHV. The liver lesions described in mice by Olitsky and Casals (1946) were detected histologically and did not make the mice evidently ill.

That MHV might be a variant of some already known virus with exaggerated hepatotropism must be reckoned as a possibility. Ectromelia produces liver lesions having a very close macroscopic resemblance to those of MHV ; lymphocytic choriomeningitis and mouse disseminated encephalomyelitis (Cheever, Daniels, Pappenheimer and Bailey, 1949) are capable of producing liver lesions. MHV is distinguished from ectromelia by many properties such as its lability, its inability to produce a local lesion on the chorio-allantoic membranes of fertile eggs and on the footpads of mice, and its failure regularly to produce evident disease in older mice. It is distinguished from LCM by its narrow host specificity, by its lability, and also by the apparent difficulty of establishing it as a neurotropic virus. This absence of neurotropism at least distinguishes it from mouse disseminated encephalomyelitis. Other points of difference are that with the agent of the latter disease no inclusion bodies have been seen, it is not present in the blood of infected mice, and it is infective for hamsters, cotton-rats and rats.

The effect of chemotherapeutic agents upon infected mice.—Treatment with terramycin and aureomycin appears to prevent the development of hepatitis in mice infected with MHV. To a lesser extent chloromycetin and sulphamerazine exert a beneficial effect, while penicillin and streptomycin seem ineffective ; streptomycin was tested both by injection and by feeding. The manner of action of these agents has not been elucidated ; present indications are that the effect is a true chemotherapeutic action on the virus. This apparent susceptibility to chemotherapeutic agents might suggest that MHV is related to the psittacosis-LGV group of viruses and, although MHV is somewhat smaller than most of this group, its size falls within the range of the group. It differs from them in that it has not been demonstrated in preparations stained by Macchiavello's method, it will not grow in the yolk-sac of fertile eggs nor does it appear to be susceptible to penicillin.

DISCUSSION.

P mice appear to carry the mouse hepatitis virus and are relatively resistant. VS mice have not yet been proved to carry it but are highly susceptible, especially when young. There is thus an analogy between a lysogenic bacterium, itself insensitive to the phage it carries, and an " indicator organism " with the aid of which presence of the phage is revealed. In so far as suckling P mice and even older ones may be killed by the virus, it is doubtful whether we should press the analogy far. The cause of the greater resistance of P mice might be constitutional, or it might be consequent upon an acquired active immunity. The increased resistance of VS mice with age is less likely to depend upon an acquired active

immunity, since virus has not been recovered from this strain. The virus seems to multiply in adult VS mice, and often kills heavily pregnant animals. This suggests that the metabolic status of mice may be related to the symptoms observed in them.

Of special interest in relation to human hepatitis is the finding that apparently normal mice may carry the virus in the blood. We have already pointed out that in several respects, notably susceptibility to ether and to heat, mouse hepatitis virus differs from human hepatitis viruses.

The circumstances of discovery of the virus and some later observations suggest the possibility that the virus may play a part in some necrotic liver lesions in mice, normally attributed to infection with *B. piliformis* or other bacteria.

Finally, the unexpected finding of the preventive action of aureomycin and terramycin, taken together with earlier work on grey lung virus from this laboratory (Andrewes and Niven, 1950) shows that susceptibility to chemotherapy is not a property confined to the psittacosis group of viruses.

SUMMARY.

1. A virus (MHV) is described causing a fatal hepatitis in young mice.
2. MHV was present in the liver, blood, kidneys, faeces and urine of infected mice.
3. The virus differs from human hepatitis viruses in its lability and the short incubation period of the infection.
4. One strain of mice (VS) was more susceptible to overt disease than another strain (P). The C3H and C57 strains appear to resemble the P strain in this respect.
5. Serial passage has activated latent MHV from the liver, kidneys, blood and faeces of apparently normal mice of the less susceptible P strain.
6. Infections with MHV were prevented by aureomycin and terramycin. Chloromycetin and sulphamerazine exerted a small effect; while penicillin and streptomycin exerted no effect under the conditions investigated.

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THE SUSCEPTIBILITY OF RATS TO ENCEPHALOMYOCARDITIS VIRUSES.

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IT has long been recognized that young animals are more susceptible than adults to certain virus infections. This susceptibility has been thought to be due to the fact that the adult is more likely than the young to have acquired a specific immunity. There is growing evidence, however, that this difference in susceptibility may be due, not to acquired immunity, but to certain metabolic peculiarities of the young, or possibly in some cases to increased permeability of certain tissues. Differences in susceptibility between the chick embryo and the hatched chick are well known, and the variation in susceptibility of the human foetus and the adult to the virus of German measles is a matter of considerable practical importance (Gregg, 1941). Theiler (1930) observed that very young mice die with encephalitis when inoculated intraperitoneally with a neurotropic strain of yellow fever virus, whereas adult mice are resistant to such an inoculation unless the brain is traumatized while virus is present in the blood stream. Whitman (1943) showed that whereas the majority of mice under 21 days of age died when inoculated intraperitoneally with neurotropic yellow fever virus, those older than 21 days survived.

Duffy and Sabin (1942) found that 7- to 8-day-old albino rats are uniformly susceptible to intracerebral inoculation of St. Louis encephalitic virus, whereas 21-day-old rats uniformly fail to develop infection. Duffy (1951) reported similar findings with Japanese B encephalitis virus in the rat, Rats aged 21 to 27 days failed to develop infection, whereas 7-day-old rats succumbed to infection when the virus was injected intraperitoneally or intracerebrally or was instilled intranasally. In the present communication the susceptibility of young rats to viruses of the encephalomyocarditis group is demonstrated.

AGE AND SUSCEPTIBILITY TO ENCEPHALOMYOCARDITIS VIRUSES.

Two strains of encephalomyocarditis virus were studied, Columbia SK virus and Senger virus ; the latter virus was isolated in Germany by Professor Hans Bielling, apparently from a human source. The inocula consisted of a 10 per cent suspension of mouse brain in physiological saline.

Experiments with Columbia SK virus.

Experiment 1.—A litter of 9 suckling rats, three days old, was inoculated with a 10 per cent suspension in physiological saline of mouse brain infected with Columbia SK virus. Four mice received 0·1 ml. of the suspension intra-

cerebrally, while 5 mice were given 2 drops intranasally. Six adult mice were inoculated intraperitoneally with 0·1 ml. of the same suspension of infected mouse brain. These mice exhibited symptoms of paralysis in 2 to 5 days. The suckling rats injected intraperitoneally were all paralysed in 4 days and were killed moribund 24 hours later. The suckling rats infected intranasally gave the following results: One rat was sick on the 4th day and was killed; one rat was dead in 7 days, and the remaining 3 rats were paralysed on the 7th day and died within 24 hours.

Blood and brain from a rat inoculated intraperitoneally 4 days previously were injected intraperitoneally into normal mice. The mice received either 0·1 ml. of blood or 0·1 ml. of a 10 per cent suspension of rat brain in physiological saline. Mice died in from 3 to 5 days with paralysis. Similar inoculations into mice were made from the brain and blood of suckling rats dying after intranasal instillation of virus. Here again mice inoculated intraperitoneally with blood and brain suspension died in from 3 to 5 days.

Experiment 2.—A litter of 8 suckling rats aged 12 days was inoculated as before, 4 being injected intraperitoneally and 4 receiving the virus intranasally.

All the rats died, those inoculated intraperitoneally in from 4 to 5 days, and those intranasally in from 5 to 8 days.

Experiment 3.—A series of rats from 22 to 25 days old was inoculated as before, 5 receiving intraperitoneal injection of virus and 4 intranasal instillation. Three of the rats which had been injected intraperitoneally died in 4 to 7 days while 2 survived; one of the rats receiving the virus intranasally died on the sixth day after injection. In both the above experiments virus from rat brain killed mice with paralysis when 0·1 ml. of a 10 per cent suspension was inoculated intraperitoneally.

Experiment 4.—A series of 8 rats, 35 to 40 days old, was injected intraperitoneally and intracerebrally. All survived and none showed any symptoms.

Proof that the virus which killed the rats up to 22 to 25 days after birth was in fact the Columbia SK virus was obtained from histological examination of the rat brains, from neutralization tests, and from sheep red-cell agglutination. The histological appearances, which will be described in detail elsewhere, were similar in character to those found in mice inoculated intraperitoneally or intranasally with the Columbia SK virus. Similar pathological changes were found in the brains of mice infected with the rat-passage virus. An immune serum prepared by the injection of Columbia SK virus into the rabbit neutralized 10,000 M.L.D. of Columbia SK virus, and the rat-passage virus when tested by the intraperitoneal mouse-protection test. Finally a 20 per cent suspension of infected rat brain agglutinated sheep red cells in the same dilution as a 20 per cent suspension of mouse brain infected with Columbia SK virus. The evidence thus shows that the virus obtained from the brains of suckling rats was in fact a member of the encephalomyocarditis group.

Experiments with Senger virus.

A similar series of experiments was carried out with the German Senger virus. This virus was without effect when inoculated intracerebrally into fully grown albino rats, but histologically small foci of cells were found in the mid-brain of rats killed 7 to 9 days after inoculation.

Experiment 1.—A litter of 8 suckling rats, 4 days old, was infected with

Senger virus. Four rats were inoculated intraperitoneally with 0·1 ml. of a 10 per cent suspension in saline of mouse brain infected with Senger virus; four suckling rats received two drops of the same suspension instilled intranasally. The rats inoculated intraperitoneally were moribund in from 2 to 3 days, while the rats which were infected intranasally developed symptoms in from 2 to 3 days and died 24 hours later.

Blood and a 10 per cent suspension of rat brain were inoculated in a dose of 0·1 ml. intraperitoneally into adult mice. Of 4 mice inoculated with blood, only 2 died in 5 and 8 days; but 4 mice inoculated with brain suspension all died, in 2, 2, 3 and 5 days.

Experiment 2.—A litter of 10 rats, aged 12 days, was inoculated intraperitoneally and intranasally with a similar 10 per cent suspension in saline of mouse brain infected with Senger virus. The five rats inoculated intraperitoneally died in from 2 to 4 days, while those infected intranasally died in from 3 to 7 days. As before, blood and a 10 per cent suspension of rat brain were injected intraperitoneally into adult mice. The series of 4 mice inoculated intraperitoneally developed paralysis in from 5 to 3 days, while those injected with rat-brain suspension died in from 3 to 5 days.

Experiment 3.—A litter of 8 rats, aged 21 days, was inoculated intracerebrally with 0·03 ml. of a 10 per cent suspension in saline of mouse brain infected with Senger virus. Seven rats died in 48 hours and 1 in 72 hours. Eight rats were injected intraperitoneally with 0·1 ml. of the same suspension. All the rats died in from 3 to 4 days. Two series of mice were injected with blood and a 10 per cent suspension of infected rat brain from a rat that had been infected intraperitoneally. The mice receiving rat blood intraperitoneally exhibited paralysis in 4 to 5 days and died in 24 hours, while the mice similarly injected with rat brain exhibited paralysis in from 2 to 4 days.

Experiment 4.—A litter of 7 rats, aged 35 days, was injected with 0·2 ml. of a 10 per cent suspension in physiological saline. One rat was found dead in 4 days, but the remainder showed no abnormal symptoms. Five rats of the same age were inoculated intracerebrally with 0·05 ml. of the same suspension. All the rats remained in good health without exhibiting any symptoms.

Proof that the virus causing the death of rats up to 22 days of age was a member of the encephalomyocarditis group and not an intercurrent virus obtained from the rats was again obtained from the histological changes found in the rat brains, from neutralization by Columbia SK immune serum, and from the specific agglutination of the sheep red cells by the virus suspension in saline. Evidence that Senger and Columbia SK viruses are closely related immunologically will be presented in a further communication.

DISCUSSION.

The rat is normally considered to be insusceptible to a considerable number of viruses, and in adult rats Columbia SK and Senger viruses cause no symptoms, although the virus persists in the brain for 13 days (Findlay and Howard, 1951). The evidence here brought forward shows that young rats up to 22 days in age are extremely susceptible to members of the encephalomyocarditis group of viruses. These observations agree with the age susceptibility of the rat to Japanese type B encephalitis and St. Louis encephalitis virus (Duffy and Sabin, 1942; Duffy, 1951). With all these viruses the critical period in the life of the

rat is from 22 to 25 days. As all these viruses have neurotropic potentialities, it is of interest to note that certain other viruses exhibit a similar limiting period. Two strains of Coxsackie virus have been trained to infect mice 22 to 24 days old, but not 30 to 35 days old. We have found similarly that the viscerotropic strain of Rift Valley fever virus can produce fatal results in rats up to 12 to 20 days of age (Findlay and Howard, unpublished), although adult rats are quite resistant. Increase in the permeability of the blood-brain barrier is thus not the only factor concerned in the susceptibility of young rodents to certain viruses to which the adult is resistant. It would seem probable that there is some profound metabolic change which occurs in young rodents at about the fourth week of extra-uterine life and that it is this change which decreases their susceptibility. Attempts to infect suckling rats with Lansing or MEF 1 poliomyelitis virus have now been successful; results will be reported elsewhere.

SUMMARY.

Suckling rats and young rats up to the age of 22 to 25 days are highly susceptible to encephalomyocarditis viruses injected intraperitoneally or intracerebrally or instilled intranasally.

Above the age of 25 days rats show no clinical symptoms of infection.

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